The c-type cytochromes of *Shewanella putrefaciens*

by

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Presented for the degree of PhD

University of Edinburgh

This work was made possible by the collaboration between the University of Edinburgh and the Torry Research Station, Aberdeen

February, 1987

For my parents

ACKNOWLEDGEMENTS

I would like to thank Dr. F. B. Ward and Dr. D. M. Gibson for their helpful criticisms and advice throughout this project, and Professor J. F. Wilkinson for permitting me to work in his Department.

Thanks are also due to Dr. G. W. Pettigrew of the Royal (Dick) School of Veterinary Medicine for doing the amino acid analyses and for invaluable advice; to Dr. Lynn Leadbeater for performing the gel permeation HPLC; to Mr. Ahmed Zahran for help with gel permeation chromatography, and to the Deacon boys (Jim, Bob, Iain, Sion and Steve), Mr. Richard Lightcap, AA, BA, MEd and Ralfie and Bertie for after hours symposia.

Special thanks are offered to Mr. T. G. Evans of G. & T. Evans, Adfa, who gave me the confidence to go for it.

SUMMARY

The respiratory system of *Shewanella putrefaciens* (*Alteromonas* sp. NCMB400) was investigated, with particular emphasis on the *c*-type cytochromes. The cytochromes present in fractions from cells grown under a variety of respiratory conditions were compared.

At least four low-potential *c*-type cytochromes were found in microaerobically grown cells and oxygen-limited cells. All appeared to function in respiration from formate to TMAO. These were identified as Peaks 4-8 by ion exchange chromatography. Flavocytochrome (Peak 4) and the cytochromes in Peaks 7 and 8 had spectra similar to cytochrome c_3, and each had multiple haem groups tentatively resolved at about -200 and -300 mV. The flavocytochrome had six haem groups and a single noncovalently-bound flavin per 89 000 d polypeptide, and reacted with CO and CN^-. The reaction with CN^- differed from that reported for phototrophic bacterial flavocytochromes, which oxidise sulphur compounds. The amino acid composition of the flavocytochrome was similar to that of the hexahaem c_{552} nitrite reductase of *E. coli*. However, no nitrite reductase, sulphide, sulphite or thiosulphate oxidase activity was associated with the flavocytochrome.

The Peak 8 cytochrome had a molecular weight of 32 000 d by gel permeation HPLC. It did not react with PAGE blue or Lowry protein reagents, and haem was

detected at the dyefront on SDS-PAGE gels. It was reactive with CO and CN^-. Amino acid analysis revealed that the cytochrome contained cysteine residues which were not involved in haem attachment. The cytochrome released hydrogen sulphide on treatment with mineral acid indicating the presence of iron-sulphur prosthetic groups.

Microaerobically-grown cells contained high-potential cytochromes $\underline{c}_{552}$ and $\underline{c}_{552,548}$ present at about 10% of total cytochromes. These were not involved in TMAO or fumarate respiration and could not be distinguished from the high-potential cytochromes found in aerobically-grown cells. Cytochrome $\underline{d}$ and high-potential cytochrome $\underline{b}$, inactive in TMAO respiration, were also detected. Two high-potential $\underline{c}$-type cytochromes were partially purified and characterised. One, designated Peak 3 in ion exchange profiles, was located largely in the periplasmic fraction of aerobic and microaerobic cells. It had a molecular weight of 8 500 d and a redox potential of +217 mV. The α-band of the reduced cytochrome was 552 nm and Peak 3 contributed about 50% of the high potential soluble cytochrome $\underline{c}_{552}$. The other high-potential cytochrome was located largely in the membrane, but could be partially released by treatment with EDTA. It had a molecular weight of 20 000 d and an absorption spectrum with an unusually small Soret band in the ferric state.

The respiratory inhibitors HQNO and Antimycin A were ineffective at interrupting electron flow between the cytochromes in either aerobic or microaerobic cells, but inhibited electron flow between NADH or formate and the cytochromes. Oxidation of the cytochromes by TMAO was inhibited by the thiol group reagent PCMBS indicating that the TMAO reductase contained iron-sulphur. Formate reduction of membranes from aerobic cells in the presence of PCMBS resulted in reduction of cytochrome $\underline{b}_{560}$ before $\underline{c}_{552}$ indicating an electron transport sequence of cyt$\underline{b}_{560} \longrightarrow$ FeS $\longrightarrow$ cyt$\underline{c}_{552}$.

The properties of these cytochromes are compared with several well-characterised bacterial cytochromes, and hypothetical schemes for electron transport in aerobically-grown and microaerobically-grown cells of S. putrefaciens are presented.

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ABBREVIATIONS

AA	Antimycin A
A26D	Anthraquinone-2,6-disulphonate
A2S	Anthraquinone-2-sulphonate
BV	Benzyl viologen
DAD	Diaminodurol
DCPIP	2,6-Dichlorophenolindophenol
DMSO	
DQ	Duroquinone
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FDH	Formate dehydrogenase
FeS	Iron-sulphur prosthetic group
FIC	Ferricyanide
FMN	Flavin mononucleotide
FR	Fumarate reductase
HNQ	Hydroxynaphthoquinone
HQNO	2-n-heptyl-hydroxyquinoline-N-oxide
MB	Methylene blue
MK	Menaquinone
$MV^{\cdot +}$	Methyl viologen, reduced
MV^{++}	Methyl viologen, oxidised
NADH	Nictotinamide adenine dinucleotide, reduced
NANA	Nutrient agar containing 2% NaCl

NQNO	2-*n*-nonyl-hydroxyquinoline-N-oxide
NR	Nitrate reductase
o/n	Overnight
Pa. denitrificans	*Paracoccus denitrificans*
PCMBS	*p*-chloromercuribenzene-sulphonate
PES	Phenazine ethosulphate
PMS	Phenazine methososulphate
T-buffer	10 mM Tris.HCl pH 8.4
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
Tris	Tris(hydroxymethyl)-aminomethane
UQ	Ubiquinone

Abbreviations of units are defined according to The Symbols Committee of The Royal Society (1975).

CHAPTER 1.

INTRODUCTION

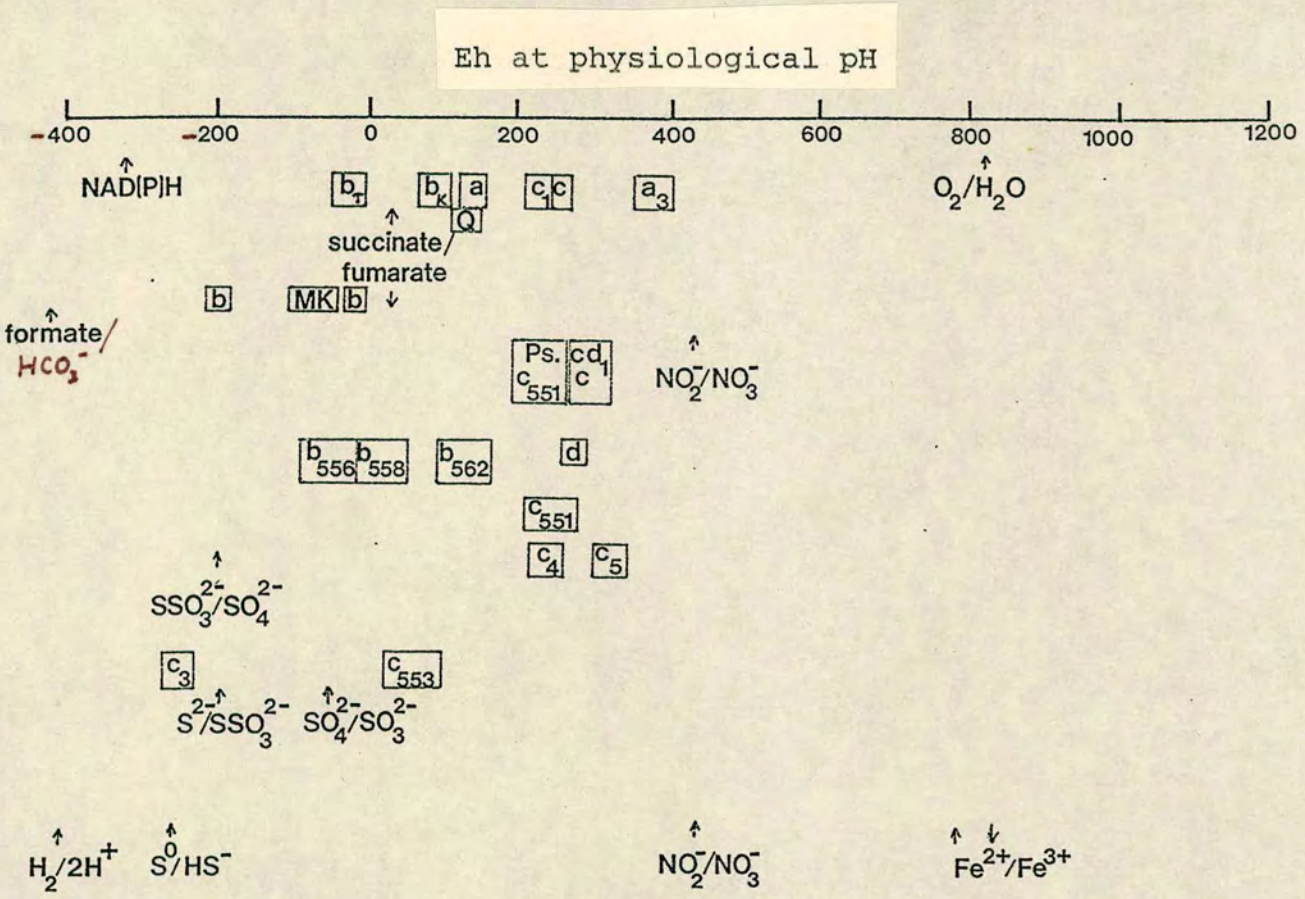
1.1 General Introduction

The ability of microorganisms to survive and reproduce in a multitude of habitats from the rumen to salt lakes is a function of their metabolic diversity: the capacity to generate energy for growth and replication in many of these environments is a reflection of the unique competence amongst the bacteria to use several alternatives to oxygen for respiration, and in many cases to switch to and from oxygen at will according to the demands of the environment. Since the occurrence and importance of bacteria in their habitats is measured in terms of their metabolic requirements and biochemical activities, a degree of physiological flexibility might reflect in the competitive ability of an organism.

With the exception of fumarate respiration by certain lower Eukaryotes (Kroger, 1977), anaerobic respiration is confined to the facultatively anaerobic and some photosynthetic and strictly anaerobic bacteria where it is exploited as a more efficient means of energy conservation than fermentation. The different modes of aerobic and anaerobic respiration available to bacteria, with some representative genera, are presented in Fig. 1.1, which also illustrates to some extent the correlation between general physiology and habitat.

The microbial flora of marine fish caught in

Fig. 1.1 Respiratory electron donors, carriers and acceptors



Aerobiosis:
mitochondria,
Pa. denitrificans

Facultative anaerobes
and denitrifiers:
Wolinella succinogenes,
Pseudomonas spp.
E. coli

Anaerobiosis:
Sulphate reducers
Desulfovibrio spp.

Chemolithotrophy:
Iron, hydrogen, sulphide
oxidising bacteria
Thiobacillus spp.
Nitrobacter

temperate water consists primarily of Gram-negative bacteria. During storage at chill room temperatures the flora alters and bacteria belonging to the genera Pseudomonas, Psychrobacter, Shewanella, Vibrio and Flavobacterium develop. Of particular importance are Shewanella spp. which have been shown to cause the characteristics of fish spoilage in model systems (Herbert et al., 1970).

The taxonomic position of Shewanella putrefaciens (formerly Alteromonas putrefaciens) has been uncertain for a number of years due to the heterogeneity of the Alteromonas group as originally defined (Baumann et al., 1972; Lee et al., 1977) and the lack of concerted analysis of these bacteria by modern methods of nucleic acid analysis (DNA/DNA and DNA/RNA hybridisation, 16s rRNA oligomer cataloguing and 5s rRNA sequencing). However, the 5s rRNA sequencing of Vibrio and related strains (MacDonell & Colwell, 1985) and the DNA/rRNA hydridisation studies on Alteromonas species now allow a clearer view of the taxonomy of these species.

Van Landschoot & DeLey (1983) classed Alteromonas species into four RNA groups:

1. Alteromonas macleodii
2. Alteromonas haloplanktis
3. Alteromonas putrefaciens
4. Marinomonas (A. communis and A. vaga).

The first two groups fell clearly into the first rRNA superfamily as defined by DeLey (1978) and were not

sufficiently dissimilar to warrant placing in different genera. They concluded, however, that Alteromonas putrefaciens should be placed in a separate genus. These results were confirmed by MacDonell & Colwell (1985) who sequenced the 5s rRNA of A. putrefaciens ATCC 8071 (NCIB 10471) and recommended that it be placed in a separate genus, Shewanella. A. vaga and A. communis were part of the second rRNA family and were so different from the other alteromonads that they were placed in a separate genus Marinomonas, part of a different family (Fig. 1.2).

Both the above studies placed the genus Shewanella as being more closely related to the Vibrionaceae than the Enterobacteriaceae but in the same major group, rRNA superfamily 1. These results are in very good agreement with those of Woese et al. (1985) who placed A.putrefaciens DSM 20456 between the Vibrionaceae and the Enterobacteriaceae in the gamma subdivision of the purple bacteria.

Fortunately the rRNA superfamily 1 of Van Landschoot & DeLey is approximately equivalent to the gamma subdivision of the purple bacteria. So the taxonomic position of Shewanella relative to other eubacteria can be summarised as shown in Figs 1.3 and 1.4 based on the phylogenetic schemes of Gibson et al. (1979), Fox et al. (1980) and Woese et al. (1985).

The psychrophilic Gram negative Shewanella putrefaciens has been isolated from a wide variety of

Fig. 1.2 Taxonomy of Shewanella, Alteromonas and Marinomas

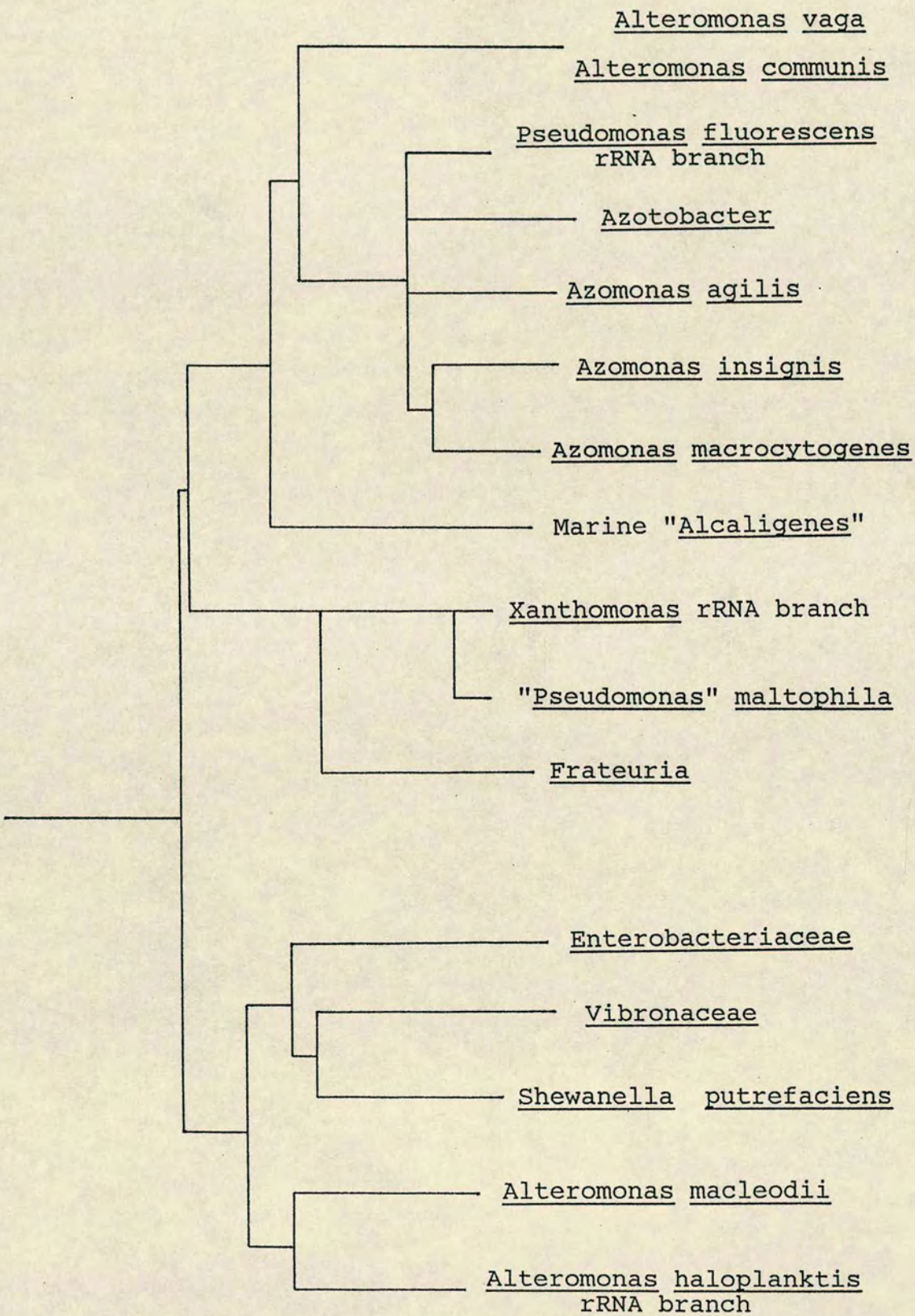
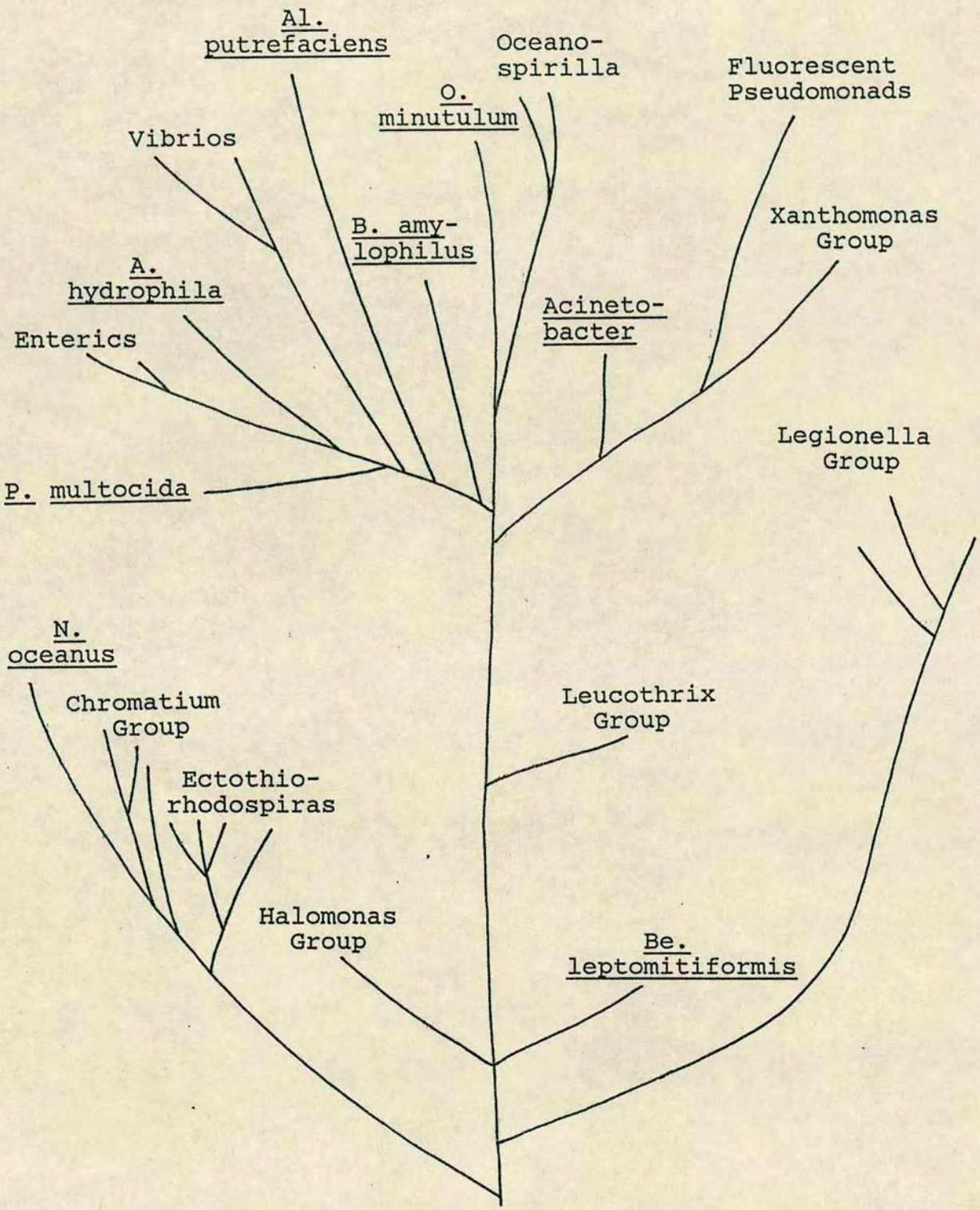


Fig. 1.3 Taxonomy of Shewanella I: gamma subdivision of the purple bacteria

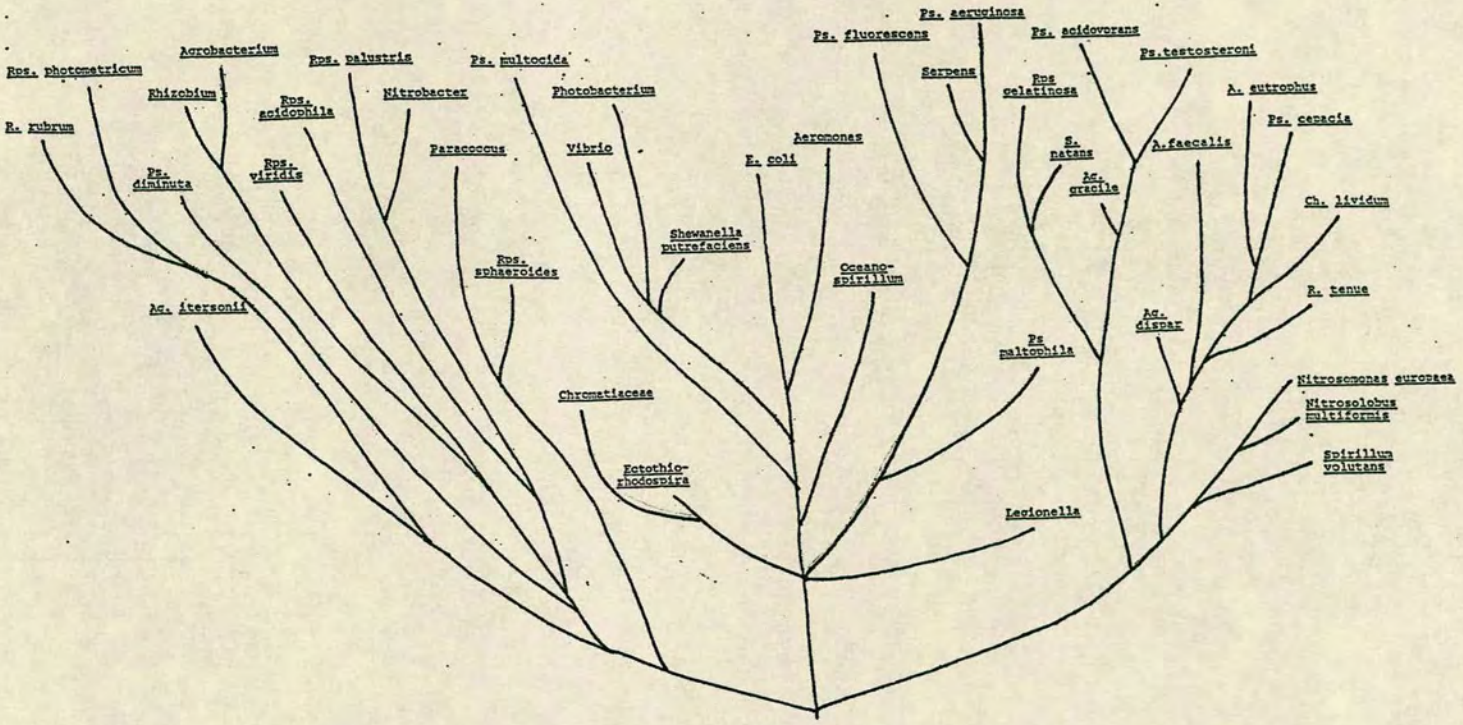


A: Aeromonas Al: Alteromonas B: Bacteriodes

Be: Beggiatoa N: Nitrosococcus

O: Oceanospirillum P: Pasteurella

Fig. 1.4 Taxonomy of Shewanella II: the eubacteria



Abbreviations:

A - Alcaligenes Aq - Aquaspirillum Ch - Chromobacterium E - Escherichia

Ps - Pseudomonas Rps - Rhodopseudomonas R - Rhodospirillum S - Sphaerotilus

proteinaceous foods (Lee, 1979; Parker & Levin, 1983) but is primarily associated with the marine environment, and is isolated routinely from the surface flora of commercially caught fish. It has been shown (Easter, 1982) that all of the bacteria with which S. putrefaciens have been grouped (Phenon D; pseudomonas-like bacteria: Lee et al., 1977) are facultative anaerobes with the ability to reduce trimethylamine-N-oxide (TMAO), a metabolite found commonly in marine organisms.

It has long been known that trimethylamine (TMA) is one of the major metabolites contributing to the odour of spoiling fish but the involvement of bacteria in the reduction of TMAO to TMA, hypothesised by Suwa in 1909, was not demonstrated until 1937 (Suwa, 1909; Beatty & Gibbons, 1937). It is now thought that the TMAO-reducing capability of facultative anaerobes may not only confer upon them a competitive advantage during fish spoilage, but may through the process of oxygen utilisation followed by TMAO consumption, derive an environment which is then suitable for true anaerobes to flourish (Easter, 1982). The importance of this process in the organoleptic deterioration of fish tissues has been considered secondary in relation to the endogenous metabolism of TMAO to dimethylamine and formaldehyde in fish tissues by Banda & Hultin (1983): however, Shewanella (Alteromonas putrefaciens) NCMB1735 and S. putrefaciens remain the subject of

investigation as important constituents of the fish spoilage flora (Section 1.1.2).

The source of TMAO in marine fish and molluscs is still not fully understood, despite the presence of concentrations over 1% of wet weight in many instances (Shewan, 1951). Some zooplankton and marine fish can oxidise TMA to TMAO (Strom, 1979), where the source of TMA may be marine algae. TMA itself is known to be a degradation product of choline and betaine, attributed to marine crustaceans (Billinski, 1962) and some Enterobacteriaceae (Dyer & Wood, 1947). Suggested roles of TMAO in marine fish include osmoregulation, analogous to the utilisation of urea by the elasmobranchs, and freezing-point depression of body fluids (Bickel, 1969; Groninger, 1959). TMAO is an oxidised ammonia analogue $(CH_3)_3N{=}O$ with the nitrogen atom exhibiting the valency of 5. Facultatively anaerobic bacteria which are capable of growth on TMAO as an oxidising agent are known to reduce the compound to TMA-$(CH_3)_3N$- with a concomitant decrease in the valency of the nitrogen atom to 3.

The utilisation of TMAO and TMA by bacteria reveals three distinct processes (Barrett & Kwan, 1985). Methylotrophic bacteria may use both compounds as sources of carbon nitrogen and energy (Boulton & Large, 1977; Large et al., 1972; Meiberg et al., 1980; Myers & Zatman, 1971; Colby & Zatman, 1973; Large, 1971). Methane derived from TMA has been

observed in certain methanogens, especially as members of consortia (Hippe et al., 1979). Secondly, TMAO may be used as an accessory oxidant in dark anaerobic growth of purple nonsulphur bacteria where the oxidation of reduced pyridine nucleotides by TMAO permits more complete and efficient oxidation of substrates such as fructose and lactate to acetate and CO_2 (Yen & Marrs, 1977; Cox et al., 1980). Thirdly, TMAO may be used as an oxidant for anaerobic respiratory electron transport.

The bacterial genera which are capable of TMAO reduction rather than assimilation are marine genera such as Alteromonas, Vibrio and Photobacterium (Gillespie, 1981; Easter et al., 1982; Lerke et al., 1965; Unemoto et al., 1965; Spencer, 1955), purple nonsulphur bacteria of the genera Rhodopseudomonas and Rhodospirillum, several members of the Enterobacteriaceae and possibly also the enteric Campylobacter (Park et al., 1980). It is noteworthy that all microorganisms which are known to be capable of anaerobic respiration with TMAO as terminal electron acceptor also have the ability to utilise nitrate: the latter is often an inhibitor of TMAO induction and both nitrate and oxygen cause a TMAO "sparing effect" (Watson, 1939), i.e. act as preferential electron acceptors (Easter, 1982). Anaerobic respiration-supported oxidative phosphorylation dependent upon oxidation of the respiratory chain by

TMAO has been demonstrated in E. coli (Takagi et al., 1981), Proteus (Stenberg et al., 1982) and Shewanella (Alteromonas putrefaciens) NCMB1735 (Stenberg et al., 1984): anaerobic growth-yield experiments with Salmonella (Kim & Chang, 1974) suggest that this organism is also capable of deriving energy from the oxidation of TMAO by a respiratory process.

Little is known of respiration to TMAO in Proteus and Salmonella. Formate, NADH and lactate reduced TMAO in Proteus (Stenberg et al., 1982; Strom & Larsen, 1979; Strom et al., 1979). The respiratory chain to TMAO is inhibited by HQNO and is reported to contain cytochromes of the b-type but not c-type (Stenberg et al., 1982). Rather more is known of TMAO respiration in Salmonella. Glycerol, formate and lactate support TMAO reduction, but not NADH (Kwan & Barrett, 1983): formate appears to be degraded by formate hydrogenlyase rather than formate dehydrogenase since FDH mutants grow well on TMAO, but formate hydrogen lyase mutants do not (Barrett & Riggs, 1982; Barrett et al., 1984; Kwan & Barrett, 1983). Electron transport components in Salmonella include flavoprotein (FMN) and menaquinone; menaquinone mutants which have TMAO reductase ability linked to exogenous electron donors cannot reduce TMAO in vivo (Kwan & Barrett, 1983). Several membrane-bound enzymes capable of reducing TMAO have been identified, one of which is constitutive, the rest inducible. The major inducible enzyme has been

purified as a high molecular weight complex probably consisting of a 332 000 d tetramer composed of 84 000 d subunits (Kwan & Barrett, 1983). Methyl viologen and FMN were good electron donors to the enzyme; cytochrome c, FAD and reduced pyridine nucleotides were not. The enzyme was inhibited by cupric ions but not KCN and did not contain haem. It probably contained the same molybdenum cofactor as nitrate reductase since molybdenum was required for the synthesis of TMAO reductase, and its activity was absent in ChlA, ChlB and ChlD mutants but could be restored in ChlD mutants by molybdenum supplmentation (Kim & Chang, 1974; Kwan & Barrett, 1983).

Much more is known about TMAO respiration in E. coli and Shewanella putrefaciens.

1.1.1 TMAO Reduction in E. coli

The anaerobic yield of E. coli grown on media containing TMAO showed that TMAO reduction was coupled to energy production providing a method of anaerobic respiration analogous to that of fumarate and nitrate respiration (Yamamoto & Ishimoto, 1977; Ishimoto & Shimokawa, 1978; Strom et al., 1979). Sakaguchi & Kawai (1975, 1977) showed that the major TMAO reductase of E. coli was membrane-bound. It was induced by TMAO and repressed by nitrate and repressed and inhibited by oxygen. Formate, lactate, reduced pyridine nucleotides

and reduced viologen dyes supported TMAO reduction, which was inhibited by the respiratory inhibitors cyanide and HQNO. Takagi et al. (1981) demonstrated proton translocation coupled to TMAO reduction in E. coli and showed that TMAO reductase and nitrate reductase shared the same molybdenum cofactor. Cox & Knight (1981) using respiration-deficient mutants of E. coli demonstrated the requirement for quinones and cytochromes for membrane-associated electron transport between NADH and TMAO. Sakaguchi & Kawai (1977) showed that HQNO inhibited oxidation by TMAO of the membrane-bound cytochromes, but not that of reduced methyl viologen.

Bragg & Hackett (1983) studied the cytochrome components of TMAO-grown E. coli in some detail, but were hindered by the presence of cytochromes of the aerobic respiratory pathway which constituted about 50% of the total cytochromes. A further 25% of total cytochromes were not involved with either aerobic or TMAO respiration. They demonstrated the oxidation by TMAO of four cytochromes, which fell into two groups on the basis of inhibition by the sulphydryl-reacting inhibitor $CuSO_4$. Reduction of the four cytochromes by formate was inhibited only slightly by HQNO, which did not affect the subsequent reoxidation by TMAO. The cytochromes could be described as high-potential on the basis of ascorbate reduction: the amount of formate- and ascorbate-reducible cytochrome oxidisible by TMAO

was almost identical. By using the impermeant oxidant ammonium persulphate, they demonstrated that the cytochromes involved with TMAO respiration were associated with the outer aspect of the cytoplasmic membrane, in contrast with the aerobic components. A tentative sequence of electron carriers has been proposed (Fig. 1.5) to integrate and explain these observations.

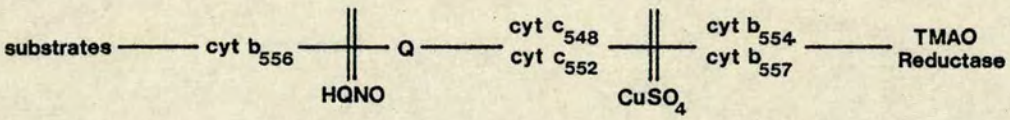


Fig. 1.5 Respiration to TMAO in E. coli

After Bragg & Hackett, 1983 and Sakaguchi & Kawai, 1977

Cytochrome b adjacent to the quinone is included in this scheme on account of the HQNO inhibition, since in E. coli the currently recognised site of inhibition is

at the level of cytochrome b or quinone, the same as for Antimycin A (Lightbrown & Jackson, 1956; Hagihara et al., 1975). Cox et al. (1970) placed the site at the level of ubiquinone before and after cytochrome b_{556} in aerobically-grown E. coli. It has been suggested that the cytochrome b oxidised by the quinone is the same as the aerobic cytochrome b (Sakaguchi & Kawai, 1977). Thus the TMAO respiratory system probably exists as a branch of the aerobic respiratory system at the level of quinone in E. coli.

A constitutive terminal reductase has been purified from the membrane fraction of E. coli and found to have a molecular weight by gel filtration of 160 000 d. It was reducible by FAD, FMN and mammalian cytochrome c but not by reduced pyridine nucleotides (Sagai & Ishimoto, 1973). The major inducible enzyme was found to have a molecular weight of about 200 000 d under nondenaturing conditions, and was resolved into 60 000 d and 80 000 d subunits under denaturing conditions. It was reduced by FAD and FMN but not by cytochrome c or reduced pyridine nucleotides (Shimokawa & Ishimoto, 1979). It is not known which, if either, of these two enzymes is active in the scheme of Hackett & Bragg. Reactivity of the constitutive enzyme with cytochrome c is in conflict with their findings since they have proposed that TMAO reductase is reduced by cytochromes b. The possibility should not be ruled out that cytochrome c, which shows considerable

cross-reactivity with structurally or functionally similar bacterial c-type cytochromes, may be substituting for cytochrome b in this system.

1.1.2 TMAO Reduction in Shewanella Spp.

Generation of a membrane potential resulting from anaerobic TMAO oxidation of formate, demonstrated by ionophore- and HQNO-sensitive serine uptake, indicates that Shewanella spp. utilise TMAO as a terminal electron acceptor for anaerobic respiration (Stenberg et al., 1984). This is supported by growth yield studies with a Shewanella sp. (formerly Alteromonas sp. NCMB1735) which showed that formate, a non-fermentable substrate, was capable of supporting anaerobic growth in the presence of TMAO (Ringo et al., 1984).

Easter et al. (1981) established that TMAO reductase in S. putrefaciens was induced by TMAO and repressed by oxygen but not fumarate or nitrate. TMAO reductase was co-induced with membrane-bound and periplasmic cytochrome c_{552}. Nasser (1983) showed that TMAO reductase, nitrate reductase and fumarate reductase were predominantly periplasmic while formate dehydrogenase was membrane-bound, which supported the conclusion of Easter (1982) that the TMAO reductase of S. putrefaciens was loosely bound to the outer aspect of the cytoplasmic membrane. TMAO reductase, nitrate reductase and formate dehydrogenase activities, but not

that of fumarate reductase, were lost in the absence of molybdenum cofactor. Complementation studies with mutants of TMAO reductase and nitrate reductase using extracts of Neurospora crassa demonstrated that the molybdenum cofactor was the same as that found in respiratory enzymes from other organisms (Nasser, 1983).

Clarke (1983) identified two TMAO reductase activities in S. putrefaciens by gel electrophoresis and zymogram staining. A 90 000 d enzyme was inducible under microaerobic conditions in the presence of TMAO or dimethyl sulphoxide (DMSO) but not with nitrate or fumarate. A 47 000 d enzyme was constitutive under microaerobic conditions. The high molecular weight enzyme was purified and characterised as a molybdoenzyme: spectral studies indicated the possible presence of flavin but no other prosthetic groups were identified.

1.2 Bacterial Respiration - An Overview

With the exception of Paracoccus denitrificans and Alcaligenes eutrophus, bacterial respiratory systems rarely show close homology with the well-characterised mitochondrial electron transport chain. A close scrutiny of the better understood bacterial respiratory systems reveals, however, that some general rules do exist which may on the one hand facilitate the study of

respiration in S. putrefaciens and on the other hand may be used to highlight any differences characteristic of this species.

1.2.1 Aerobic Respiration

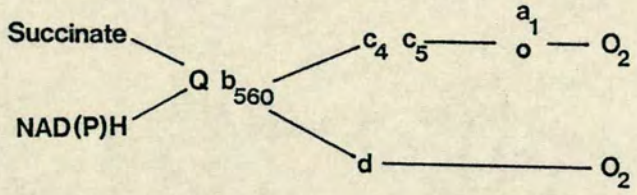
Whilst the respiratory chains of bacteria grown aerobically contain the same types of redox carriers as those present in mitochondria - FeS proteins, flavoproteins, quinones and cytochromes (including cytochrome oxidases), the most apparent feature about them is the variety of repiratory patterns displayed, and also their responses to inhibitors, which is generally less than that observed with mitochondria (Meyer & Jones, 1973a; Gel'man et al., 1975; Jones & Meyer, 1976). The variations in components between and within bacterial species, and between the bacteria and mitochondria, can usually be attributed to either the replacement of one type of carrier with another, or the addition or deletion of specific carriers (cf cytochrome c in aerobically-grown E. coli, Section 2.1.2). Variations in the pattern of redox carriers can also be induced within single species by altering the growth conditions and occur principally amongst the quinones and the terminal oxidases. Many bacteria synthesise branched respiratory pathways (White & Sinclair, 1970) or partial replacements for existing pathways under conditions of oxygen deprivation or in

the presence of low concentrations of cyanide. Branching is often present at the terminal end of the respiratory system during fumarate or nitrate respiration, since the appropriate anaerobic respiratory systems are often accompanied by significant amounts of presumably constitutive cytochrome oxidase(s) (Haddock *et al.*, 1976).

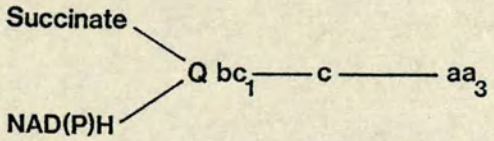
The aerobic respiratory chains of a number of bacteria which have been extensively studied are shown in Fig.1.6. The only feature common to any of these respiratory chains concerns the quinone. In most cases, the immediate electron donor is depicted as a respiratory chain-linked dehydrogenase, while the electron acceptors from quinone are usually *b*-type cytochromes, which could be considered to be analogous to the bc_1, complex of mitochondria. This arrangement has been shown to exhibit two coupling sites for oxidative phosphorylation: site 1, the (NADH) dehydrogenase, and site 2, the quinone-cytochrome *b* region, in a wide range of bacteria including *Paracoccus denitrificans*, *Azotobacter vinelandii*, *Pseudomonas ovalis* Chester, *Mycobacterium phlei*, *Alcaligenes eutrophus* and *Acinetobacter lwoffi* (Imai *et al.*, 1967; Eilermann *et al.*, 1970; Jones *et al.*, 1975; Asano & Brodie, 1965; Ishaque & Aleem, 1970). Coupling site 3 is not always present. Some bacteria which synthesise respiratory pathways which are branched at the oxidising end, such as *A. vinelandii*,

Fig. 1.6 Bacterial aerobic respiratory chains

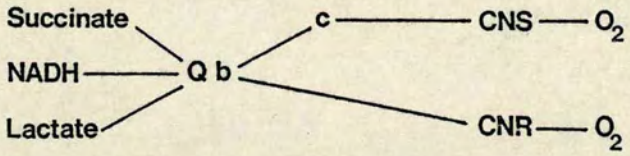
CNR = CN^- resistant oxidase; CNS = CN^- sensitive oxidase



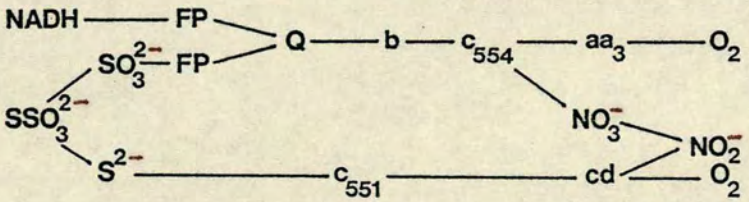
Azotobacter vinelandii



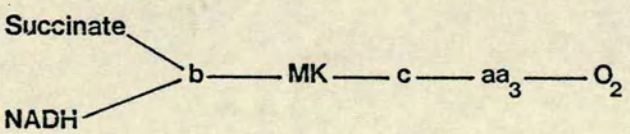
Pa. denitrificans



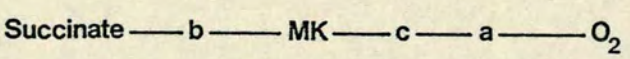
Beneckea natriegens



Thiobacillus denitrificans



Bacterionema matruchotii



Corynebacterium diphtheriae

exhibit unequal phosphorylation efficiencies depending upon growth conditions (Ackrell & Jones, 1971; Downs & Jones, 1975). Hence A. vinelandii affords protection to its nitrogenase under highly aerobic conditions by routing electron flow through the b d pathway thereby increasing the respiratory rate with a concomitant decrease in the ambient oxygen concentration (Yates & Jones, 1974).

1.2.2 Aerobic Respiration in E. coli

The aerobic respiratory system of E. coli is wholly contained within the cytoplasmic membrane, and is capable of oxidising NADH, succinate, D- and L-lactate, L-glycerol-3-phosphate, L-malate, formate, -hydroxybutyrate, dihydro-orotate and pyruvate (Haddock & Jones, 1977; Hendler, 1976; Jones, 1977; Hendler et al., 1969; Ingledew & Poole, 1984). The composition of the respiratory chain varies with the substrate and the terminal electron acceptor (Konings & Bronstra, 1977; Kroger, 1977; Haddock & Jones, 1977; Ashcroft & Haddock, 1975; Nishimura et al., 1983), a feature which is now widely recognised with many bacterial species (Broom et al., 1981; Harvey & Lascelles, 1980; White & Sinclair, 1970; Ensley & Finnertey, 1980; Liu et al., 1983; Sweet & Peterson, 1978). Spectroscopic studies with E. coli reveals cytochromes d, a_1 and b in aerobic cells: nonhaem-iron

flavoprotein and FeS protein can be detected but are analysed by different methods (Lemberg & Barrett, 1973; Fujita & Sato, 1963). Cytochromes d and o are revealed by reduced plus carbon monoxide minus reduced difference spectra (Revsin & Brodie, 1969; Bragg, 1980). Ubiquinone-8, menaquinone-8 and/or demethylmenaquinone-8 are also found in association with the respiratory chain. Ubiquinone-8 is usually found in aerobically-grown cells (Polglase et al., 1966) although menaquinone-8, usually found in anaerobically-grown cells, may be able to substitute for it under some circumstances (Wallace & Young, 1977a, 1977b).

It has been suggested that nonhaem iron present in the membranes of aerobically-grown E. coli exists as a complex with ubisemiquinone (Downie & Cox, 1978), a scheme derived from studies with a mutant unable to form ubiquinone. This would be consistent with the mitochondrial system where the bc_1, complex is known to contain a ubiquinone-binding protein (Yu et al., 1977). While there is no firm evidence for the exact site of the Fes protein, loss of site 1 associated with diminished levels of FeS protein suggests that nonhaem iron is present in the NADH dehydrogenase region of the respiratory chain (Poole & Haddock, 1975).

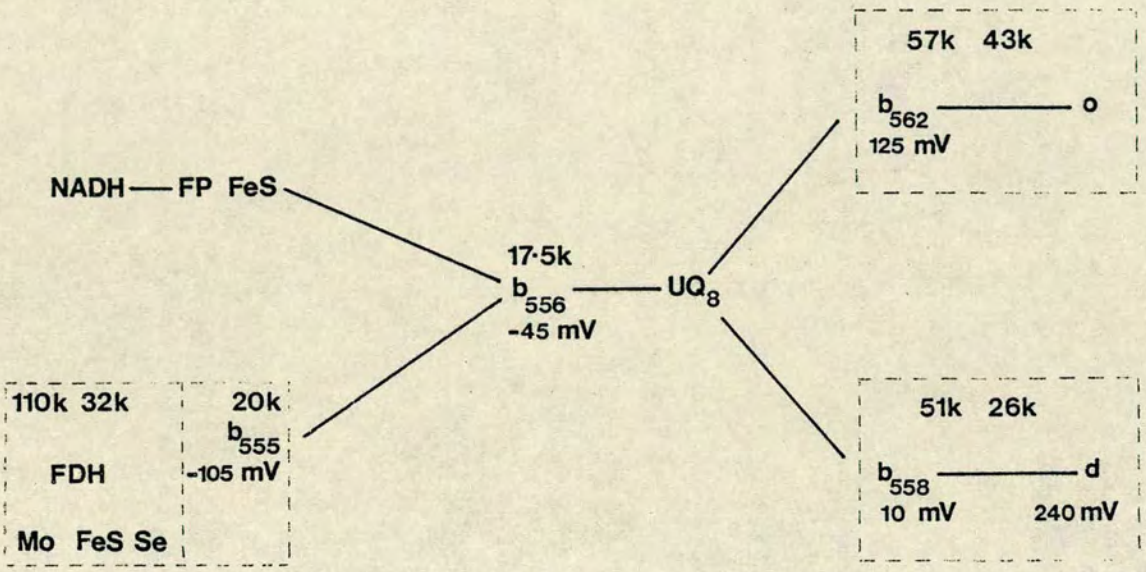
Shipp (1972a) resolved three b-type cytochromes in E. coli: b_{556}, b_{558} and b_{562}. This number was increased to four by potentiometric determinations

(Reid & Ingledew, 1979; Van Weilink et al., 1983). The position of these cytochromes in the respiratory chain, relative to the primary dehydrogenases, quinones and terminal oxidases, was attempted by Downie & Cox (1978) by spectrophotometic studies using quinone mutants. Pudek & Bragg (1976a) used the midpoint oxidation-reduction potentials of the cytochromes in conjunction with aerobic steady-state reduction levels. The aerobic respiratory pathway of E. coli was, however, only recently elucidated by purification of the bo and bd terminal oxidase complexes (Kita et al., 1984a; 1984b). A scheme has been proposed for the organisation of the aerobic respiratory components within the cytoplasmic membrane of E. coli which is quite different to the mitochondrial respiratory system: arguably, the greatest difference is the loss of coupling site III due to the absence of cytochrome c (Jones, 1977) (Fig. 1.7).

1.2.3 Anaerobic Respiration I. Respiration to Fumarate

In the absence of molecular oxygen, oxidative phosphorylation can only continue to supply the energy requirements of the cell if an alternative chemical to oxygen with a suitably high redox potential is available to oxidise the respiratory chain. Thus, bacteria are known which can reduce nitrate, nitrite,

Fig. 1.7 Aerobic respiratory chain of E. coli



(After Kita et al., 1984 and Ingeldew & Poole, 1984)

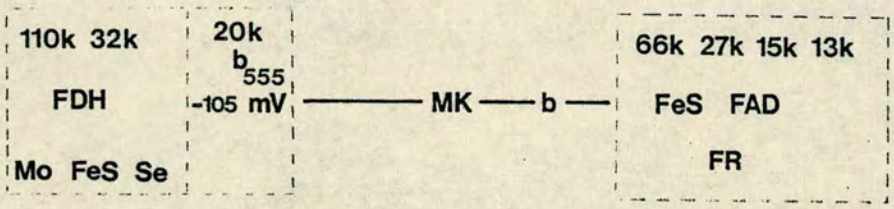
fumarate, thiosulphate, sulphur, tetrathionate, carbonate, TMAO and DMSO. Fumarate and nitrate reducing ability are equally widespread amongst the bacteria and quite common (Kroger, 1977), while some electron acceptors are only utilised by highly-specialised organisms growing in unusual and sometimes biologically extreme environments (Fig. 1.1).

A range of substrates including molecular hydrogen, formate, NADH, lactate, malate and glycerol-1-phosphate may act as hydrogen donors for the electron transport chain supporting fumarate reduction. The electron transport components are situated in the cytoplasmic membrane of most bacteria (Kroger, 1977, 1978). Electron transport to fumarate has been well-characterised in Wolinella succinogenes (Kroger, 1980) and in E. coli (Cole, 1984). Both respiratory chains are composed of the dehydrogenases, a diffusible napthoquinone and the fumarate reductase. Cytochromes of the b-type are also present. The quinone is usually menaquinone: the specificity for menaquinone rather than ubiquinone can be explained by the difference in redox potentials. Menaquinone (Eo' = -74 mV; Wagner et al., 1974) has a redox potential compatible with the succinate/fumarate couple (Eo' = . 33 mV) whereas the redox potential of ubiquinone (Eo' = 133 mV; Schnorf, 1966) is thermodynamically unfavourable for electron transport to fumarate.

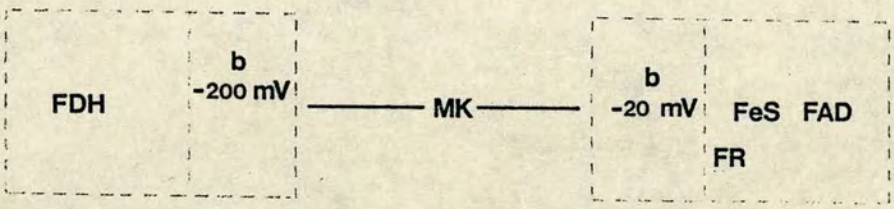
Cytochrome b is usually found in the membranes of

fumarate-respiring bacteria (Thauer et al., 1977). Inhibition of electron transport in the presence of the quinone analogue HQNO (2-n-heptyl-hydroxyquinoline-N-oxide) and its nonyl analogue NQNO is commonly interpreted as demonstrative of the involvement of b-type cytochromes, and these inhibitors block respiration to fumarate in most of the bacteria which have been studied (Thauer et al., 1977). In Wolinella succinogenes two b-type cytochromes are involved in fumarate respiration: one is a subunit of the formate dehydrogenase, the other is a subunit of the fumarate reductase. In E. coli the reduction of fumarate with NADH and glycerophosphate occurs in a cytochrome-deficient mutant (Singh & Bragg, 1976) albeit at a low rate, indicating that b-type cytochromes are not obligatory for electron transport to fumarate to occur. The formate dehydrogenase of E. coli, however, contains a b-type cytochrome as well as Se, Mo and FeS prosthetic groups (Enoch & Lester, 1975; von Jagow & Sebald, 1980). Proteus rettgeri is similar in this respect, with reduction of fumarate by formate, but not NADH, proceeding via cytochrome b. The formate-fumarate respiratory systems of W. succinogenes and E. coli are thus essentially similar (Fig. 1.8), consisting of a cytochrome b-containing formate dehydrogenase, menaquinone, fumarate reductase and a second cytochrome b. The cytochrome b_{555} responsible for electron transport from menaquinone to

Fig. 1.8 Respiratory chains to fumarate in E. coli and Wolinella succinogenes.



E. coli (after Cole, 1984 and Ingledew & Poole, 1984)



W. succinogenes (after Kroger, 1980)

fumarate reductase in E. coli is a discrete component, as opposed to that of W. succinogenes where it is a subunit of the enzyme (Ingledew & Poole, 1984).

1.2.4 Anaerobic Respiration II. Respiration to Nitrate

Two major and distinct bacterial systems for reducing nitrate are known. Nitrate may be reduced in a soluble assimilatory process for incorporation into cellular material, or it may be used as a substitute oxidant for the electron transport chain, supporting growth during conditions of oxygen deprivation. The respiratory, or dissimilatory, nitrate reductases are usually membrane-bound proteins containing MO and FeS prosthetic groups (Haddock & Jones, 1977; Stouthamer et al., 1980; Thauer et al., 1977; Payne, 1973). Nitrate reductase A (Pichinoty, 1964; E.C. 1.7.99.4) is distinguished from the soluble assimilatory enzyme B by its ability to reduce chlorate and bromate and its sensivity to azide. The purified enzymes from E. coli (MacGregor & Christopher, 1978), Paracoccus denitrificans (Forget, 1971), Klebsiella aerogenes (Van't Riet et al., 1975), Pseudomonas aeruginosa (Ferguson & Nicholas, 1961) and Neurospora crassa (Nicholas & Wilson, 1964) are all Mo, FeS proteins and are all inhibited by azide, inactivated by cyanide and not affected by carbon monoxide (Stouthamer, 1976). The best studied systems are those of E. coli and Pa.

denitrificans.

The E. coli respiratory nitrate reductase is composed of three subunits: subunit A (142 000 d) catalyses the reduction of nitrate; subunit B (60 000 d) is implicated in binding of the complex to the cytoplasmic membrane (DeMoss, 1977) and subunit C (19 500 d) is a b-type cytochrome, cytochrome b^{NR}_{556}. A stoichiometry of 2A:2B:4C has been proposed (Enoch & Lester, 1975). Electrons are obtained from quinone by the cytochrome b^{NR}_{556} at the periplasmic aspect (Boxer & Clegg, 1975; Graham & Boxer, 1978; Garland et al., 1975) as shown by labelling studies using antibodies and membrane-impermeant amino-acid specific reagents, and membrane-impermeant redox dyes. The nitrate reductase of Pa. denitrificans is also reduced at the cytoplasmic aspect of the coupling membrane (Forget & Rimassa, 1977).

Although formate acts as a more efficient electron donor than NADH to nitrate in vitro, E. coli has been shown to demonstrate preferential utilisation of NADH in the presence of formate, indicating that in vivo the source of electrons for the reduction of nitrate is NADH (Ruiz-Herrera & De Moss, 1969; Ishimoto & Yamamoto, 1977) although it is possible that with several active dehydrogenases simultaneously present in the coupling membrane, each dehydrogenase may make a contribution to the overall flux of reducing power depending on the metabolic state of the cell (Ingledew

& Poole, 1984).

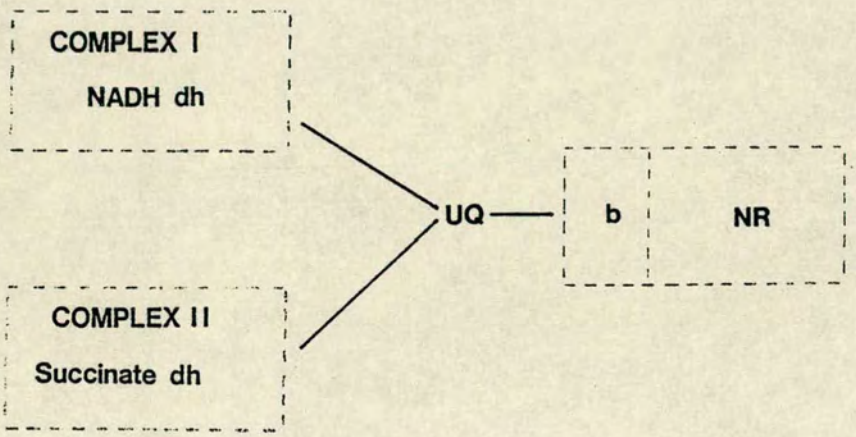
Most of the bacteria which synthesise a particle-bound nitrate reductase also synthesise a particle-bound cytochrome b, which is substrate-reducible and nitrate-oxidisible, probably via a quinone, in a reaction sequence which is inhibited by HQNO and NQNO. The quinone in Gram-negative bacteria appears to be ubiquinone, although menaquinone may substitute for it in E. coli where the rate of cytochrome b^{NR}_{556} reduction is lower than with uniquinone (Thauer et al., 1977; Enoch & Lester, 1974). Schemes for electron transport to nitrate in E. coli and P. denitrificans are given in Fig. 1.9.

1.2.5 Anaerobic Respiration III. Respiration to Nitrite

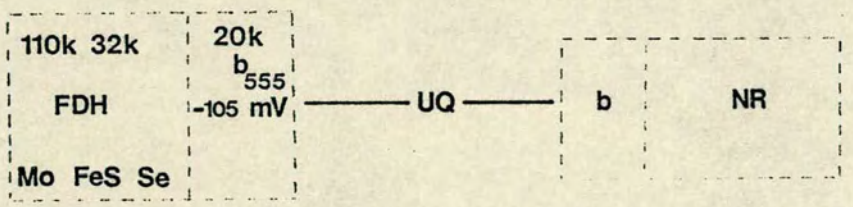
Respiratory nitrite reductases differ fundamentally from nitrate and fumarate reductase in that they are not generally membrane-bound (see however Nasser, 1983). Several types of nitrite reductase have been isolated, the best characterised examples being cytochrome cd_1 and the hexahaem nitrite reductase of E. coli. The product of reduction of nitrite differs with enzymes from different sources.

Cytochromes cd_1 consist of two identical 60 000 Mr subunits each containing one haem c and one haem d_1 (Kuronen et al., 1975; Henry & Bessieres, 1984), and

Fig. 1.9 Respiratory chains to nitrate in E. coli and Paracoccus denitrificans



Pa. denitrificans



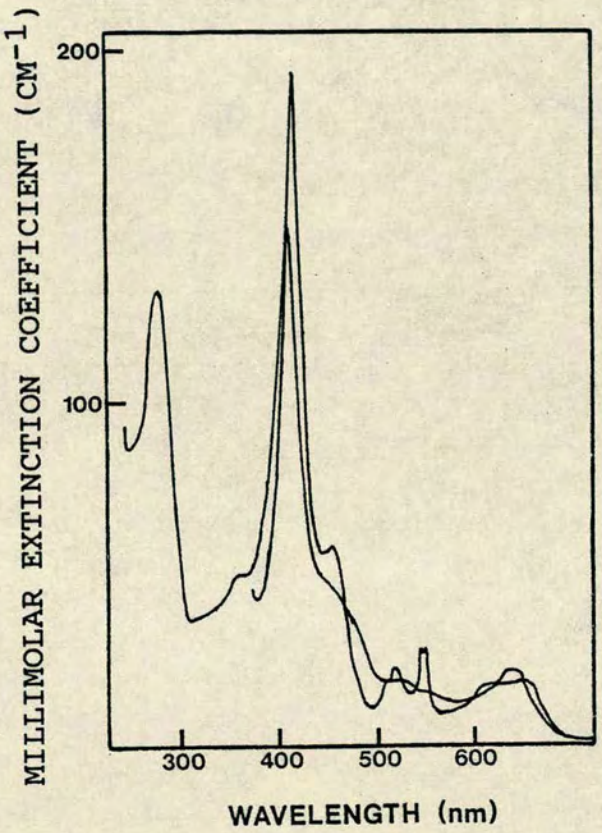
E. coli

has been purified from a range of diverse bacterial species including Pseudomonas aeruginosa (Silvestrini et al., 1983), P. perfectomarinus (Liu et al., 1983), Paracoccus denitrificans (Timkovich et al., 1982), Thiobacillus denitrificans (LeGall et al., 1979) and Alcaligenes faecalis (Matsubara & Iwasaki, 1972). The Pseudomonas enzyme may function as an oxidase under certain conditions (Hartingsveldt & Stouthamer, 1973: Timkovich & Robinson, 1979) and it is interesting to note that while the haem c is a typical low-spin his-Fe-met hexacoordinate species in both the Fe^{2+} and Fe^{3+} states, the haem d_1 changes from a low-spin his-Fe-his ferric state to a high-spin pentacoordinate state on reduction, similar in fashion to cytochrome aa_3 and P450 (Muhoberac & Wharton, 1983; Foote et al., 1984; Timkovitch & Cork, 1983; Huynh et al., 1982). The cytochrome cd_1 from Pseudomonas spp. is reduced by cytochrome c_{551} or azurin and the electrons are taken up by haem c in the nitrite reductase molecule (Ching et al., 1982).

The structure of the haem d_1 is disputed (Timkovich et al., 1984; Chang, 1985) but is known to differ from the haem d of Aerobacter aerogenes (Newton, 1969; Lemberg & Barrett, 1973). With few interspecific differences, the spectra of cytochrome cd_1 are similar to that shown in Fig. 1.10.

Energy conservation at the expense of nitrite is less efficient than with oxygen (about 60% in P.

Fig. 1.10 Spectra of cytochrome $\underline{cd}_1$



Redrawn from Meyer & Kamen, 1982

denitrificans; Koike & Hattori, 1975) and has been attributed (Boogerd et al., 1981) to the periplasmic location for the prototrophic reaction in Pa. denitrificans (Alefounder & Ferguson, 1980) and P. aeruginosa (Wood, 1978a). In E. coli the situation is less clear. A soluble NADH : nitrite reductase and a formate : nitrite reductase have both been investigated for some time. The NADH dependent nitrite reductase is a soluble cytoplasmic protein with sirohaem, FAD and FeS prosthetic groups, and it is believed to be a detoxification system since energy is not conserved at the expense of nitrite reduction (Cole & Brown, 1980). The formate-dependent enzyme utilises the same membrane-bound proton-pumping formate dehydrogenase as formate : nitrate reductase and energy is conserved in the process of nitrite reduction (Pope & Cole, 1982).

Kajie & Anraku (1986) purified a low-potential 69 000 d hexahaem cytochrome c_{552} nitrite (hydroxylamine) reductase from the periplasm of E. coli but were unsuccessful in attempts to reduce the protein with physiological substrates even in the presence of active membrane particles. Hence the significance of the protein is unclear.

Similar proteins to the haem c-containing dissimilatory nitrite reductase of E. coli are found in Achromobacter fischerii (Husain & Sadana, 1974) and Desulfovibrio desulfuricans (Liu & Peck, 1981), while a non-haem, copper-containing nitrite reductase which is

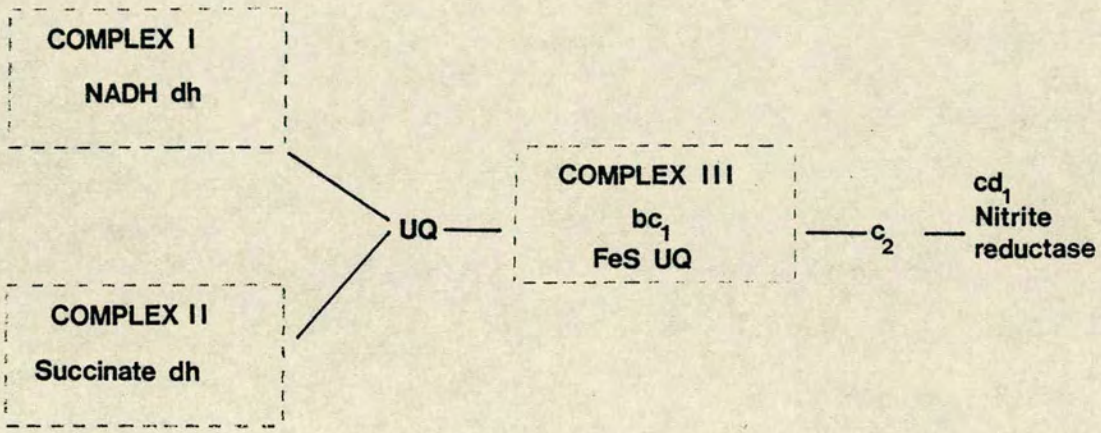
reducible by cytochrome c is found in Achromobacter cycloclastes (Iwasaki & Matsubara, 1972), Alcaligenes spp. (Miyata & Mori, 1969) and Rhodopseudomonas sphaeroides (Sawada et al., 1978).

A number of c-type cytochromes are found in both soluble and particulate fractions of denitrifying cells (Ambler, 1977a; Liu et al., 1983), the exact function often being ambiguous or totally unknown. The largely soluble cytochromes c_{551} are generally found in denitrifying bacteria as electron donor to cd_1 (Yamanaka et al., 1961). Membrane-bound cytochromes c_4 and c_5 appear alongside cytochrome c_{551} and azurin in denitrifying pseudomonads as well as in Azotobacter vinelandii where they were first characterised (Tissieres, 1956). Their function in denitrification is not understood, and in Azotobacter they are believed to act as electron carriers in aerobic respiration (Jones & Redfearn, 1967). The respiratory chain to nitrite in Pa. denitrificans is given in Fig. 1.11.

1.3 The Structure and Function of Bacterial Cytochromes

In order to elucidate the structure of the electron transport pathway from formate to TMAO in Shewanella putrefaciens the components should if possible be separated, individually identified and characterised. The evidence required to implicate a component, such as a cytochrome, in an electron

Fig. 1.11 Respiratory chain to nitrite of Paracoccus denitrificans



transfer process is as follows. Firstly, the occurrence of the component and the associated electron transfer activity should be related depending upon conditions of growth; the component may be induced in the presence of metabolites involved as electron donors or acceptors in the electron transport chain. Secondly, the component must be kinetically competent in the proposed electron transport scheme. Reactivity with proposed oxidases or reductases must be sufficient to support rates of electron transport which are observed in vivo. Finally, genetic loss of the component should have predictable metabolic consequences.

The information obtained might facilitate a tentative sequencing of the components of the respiratory chain which could be supported by established methods such as inhibitor studies and measurement of redox potentials, and might also give some indication of possible sites of energy conservation. Since the terminal enzyme of the TMAO respiratory pathway in S. putrefaciens has been purified and characterised (Clarke, 1983) and the cytochromes have not been investigated in any detail, this section will cover the cytochromes, and the variety of methods established for their characterisation and quantification. The characteristics of known and well-understood cytochromes, where possible, will be discussed, and

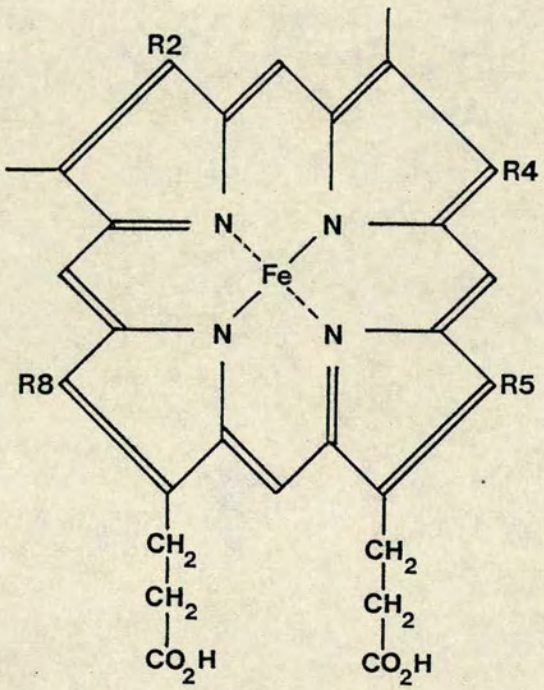
also the current system of nomenclature.

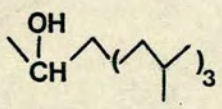
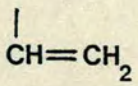
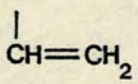
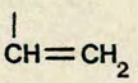
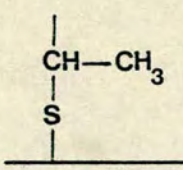
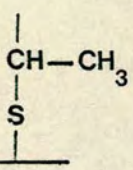
Cytochrome Nomenclature

A subcommission on cytochrome nomenclature of the International Union of Biochemistry (DerVartanian & LeGall, 1974) has recommended a system of cytochrome classification based on chemical rather than functional properties. This system requires that the haem prosthetic group of a cytochrome be characterised, thus distinguishing four major classes. Cytochromes a have noncovalently-bound haem a with a formyl sidechain as prosthetic group. Protohaem is the prosthetic group in cytochromes b and there are no covalent linkages to the protein. In the cytochromes c the haem is covalently bound to the polypeptide chain. Cytochrome d has a different type of prosthetic group called a chlorin (dihydroporphyrin).

The association of ligands with the haem iron falls into two categories. Where the extraplanar fifth and sixth coordination positions of the iron atom are occupied, the cytochrome is said to be "low-spin", and where only one coordination position is occupied the cytochrome is said to be "high-spin". The terms low- and high-spin reflect two different patterns of electronic orbital arrangement of the iron atom which are distinguished easily by the spectral properties of the cytochrome. The structures of haems a, b and c are given in Fig. 1.12.

Fig. 1.12 Structures of haems a, b and c



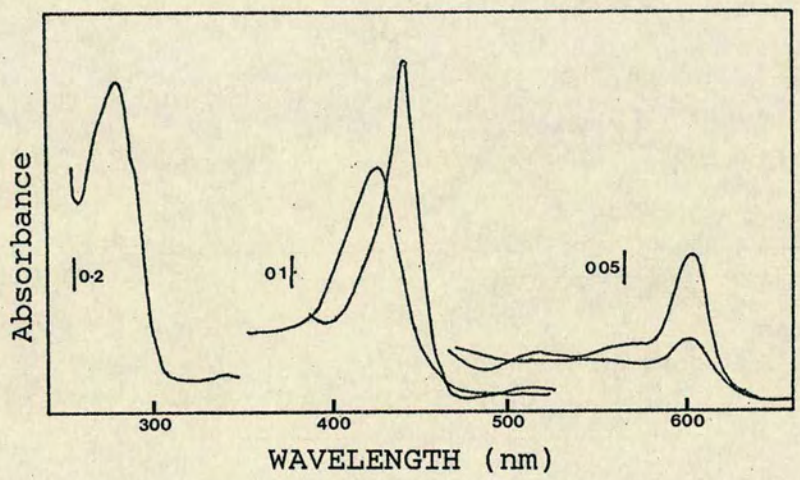
HAEM	R2	R4	R5	R8
A			—	—CHO
B			$-CH_3$	$-CH_3$
C			$-CH_3$	$-CH_3$

1.3.1 Cytochromes a

Cytochromes of the a-type contain a haem prosthetic group which has a formyl sidechain (Fig. 1.12). The haem is noncovalently bound to the protein, is readily removable from it by acid acetone extraction, and once extracted from the protein it is soluble in ether. In alkaline solutions with pyridine as the fifth and sixth field ligands of the iron atoms, the haem shows an α-peak at 580-590 nm. Bacterial a-type cytochromes can be classified into two groups according to their spectral properties in the native state. Cytochrome a_1 has α and β reduced bands at 585-595 nm and 435-445 nm respectively. Cytochromes a and a_3 from mitochondrial and bacterial sources have α and γ reduced bands at 600-605 nm and 440-445 nm respectively (Fig. 1.13). Identification of a-type cytochromes is confirmed by spectrochemical responses to the ligands CO and CN^- being similar to those of mitochondrial cytochrome aa_3 (Horio & Kamen, 1970) but this method, as well as the light-dependent relief of CO inhibition of respiration, cannot be used to differentiate between cytochrome a_1 and aa_3 because the responses are too similar (Pettigrew & Moore, 1987).

The mitochondrial cytochrome aa_3 (cytochrome oxidase) has been extensively studied and is consequently used as a reference to which bacterial cytochromes aa_3 are compared both in terms of structure

Fig. 1.13 Spectra of cytochromes <u>aa</u>$_3$



Redrawn from Weiss & Ziganke, 1978

and function. Mitochondrial cytochromes aa_3 contain seven or more subunits, two mol copper per mol complex, and one mol each of haems *a* and a_3. The complexes catalyse the four-electron reduction of oxygen to water by cytochrome *c* and pump protons using energy derived from this process, when reconstituted into proteoliposomes (Kadenbach & Merle, 1981; Hatefi, 1985).

Despite their widespread occurrence (Meyer & Jones, 1973a) few *a*-type cytochromes have been purified from bacterial sources, but those which have been characterised show strong similarities with the mitochondrial enzyme in terms of haem *a* and Cu environments and proton pumping, despite their composition of at most three nonidentical protein subunits (Ludwig, 1980; Fee *et al.*, 1980; Seelig *et al.*, 1981; Powers *et al.*, 1981; Kent *et al.*, 1982).

E. coli is known to synthesise an a_1-type cytochrome having two redox components which have been resolved by redox titrimetry into +260 mV and +160 mV components (Reid & Ingledew, 1979). Other values have been observed, however, depending upon growth conditions (Hendler & Schrager, 1979). Photochemical action spectra have also yielded conflicting results (Castor & Chance, 1959; Poole *et al.*, 1981a), as have stopped-flow kinetic studies (Haddock *et al.*, 1976) leaving the function of the cytochrome as an oxidase unproven. A protein having properties similar to

cytochrome a_1 has been purified from *E. coli*. It contained only protohaem as prosthetic group despite maxima in CO difference spectra of 440 and 590 nm and had high catalase and cytochrome *c* peroxidase activities. It was suggested that since cytochromes a_1, *d* and *b* copurify, the cytochrome a_1 may associate with cytochrome *d* in order to ensure complete reduction of oxygen to water (Ingledew & Poole, 1984).

A cytochrome oxidase has been purified from *P. aeruginosa* which contains a_1-type haem (ferrocytochrome *c* : O_2 oxidoreductase, E.C. 1.9.3.1). The reduced spectrum of the oxidase had maxima at 420, 522, 552 and 595 nm, and the properties of the cytochrome suggested that it differed fundamentally from the cd_1 nitrite reductase (Azoulay & Couchoud-Beaumont, 1965). Cytochrome a_1 has been examined in *Acetobacter* sp. and other bacteria (Poole, 1983). The cytochromes bound CO and possibly also nitrate and nitrite, and few appeared to be oxygen reactive.

In contrast with cytochrome a_1, cytochrome aa_3 has been well-characterised in a number of bacteria including *Thiobacillus novellus* (Yamanaka *et al.*, 1985), *Rhodopseudomonas sphaeroides* (Gennis *et al.*, 1982) *Paracoccus denitrificans* and the thermophilic bacterium PS3. The enzymes are simpler than the mitochondrial cytochromes in having only two or three subunits, two of which appear to be related closely to subunits I and II of the mitochondrial cytochrome

according to immunological studies and amino acid composition and sequence. Haems *a* and a_3 and two copper atoms are present which are potentiometrically similar to the mitochondrial enzyme, as are ligand-binding properties. The cytochrome aa_3 purified from the cytoplasmic membrane of *Pa. denitrificans* consisted of only two subunits of apparent Mr 45 000 d and 28 000 d by SDS-PAGE. These appeared to correspond functionally to the two largest subunits of the 7-subunit cytochrome *c* oxidise from yeast mitochondria (Ludwig & Schatz, 1980). The enzyme oxidised mammalian cytochrome *c* at a high rate and when incorporated into proteoliposomes, generated an electrochemical proton gradient during cytochrome *c* oxidation (Puttner *et al.*, 1983).

The cytochrome oxidases from *Bacillus firmus* (Kitada & Krulwich, 1984), *Thermus thermophilus* HB8 (Yoshida & Fee, 1984) and the thermophilic bacterium PS3 (Baines *et al.*, 1984; Sone & Yanagita 1984) contain bound *c*-type haem, which is associated with subunit II of the thermophilic bacterial enzymes. A consequence of this is that polarographic assay of the enzymes using TMPD as reductant does not require added cytochrome *c* as it does with mitochondrial cytochrome *c* oxidase. Indeed *Pa. denitrificans* and *Rps. sphaeroides* membranes depleted of soluble cytochrome *c* exhibit high rates of TMPD oxidation and it has been suggested that tightly-bound cytochrome *c* may be a common feature in

bacterial cytochrome oxidases (Pettigrew & Moore, 1986).

The cytochromes *c* which act as electron donor to bacterial cytochrome *c* oxidases share a number of features, in particular periplasmic location (Lorence *et al* 1981), redox potential (190-280 mV: Pettigrew & Moore, 1986), size and amino acid sequence, which apparently group them with cytochromes c_2 as "large" Class I cytochromes. Consequently, cross-reactivity between donor cytochrome *c* and acceptor oxidase from different sources even bacterial/mitochondrial, is common and may be used as evidence for a common ancestor molecule for bacterial and mitochondrial cytochrome *c* oxidases (see however Class I cytochromes, Section 1.3.3).

1.3.2 Cytochromes b

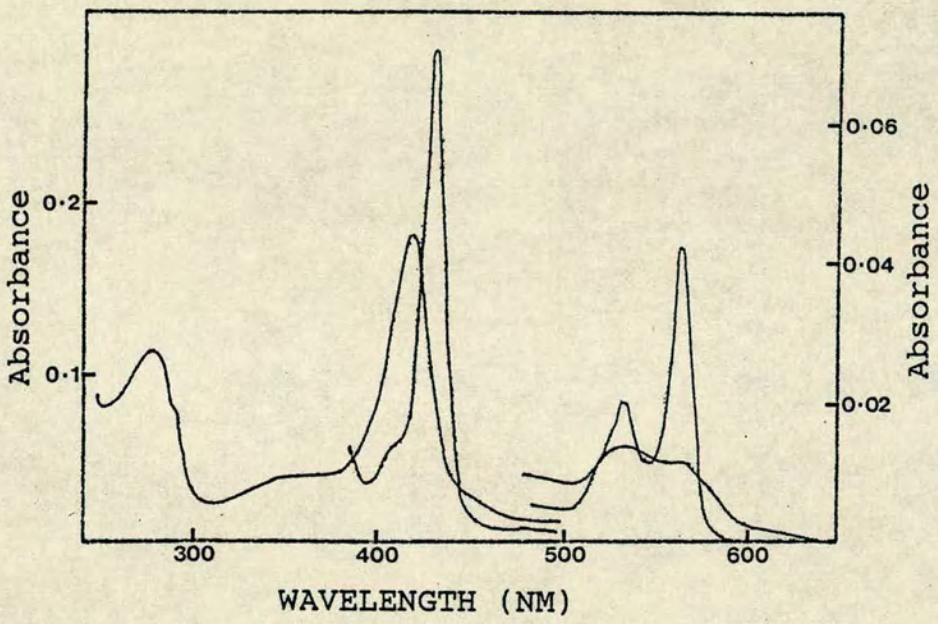
Cytochromes are placed in this category if the prosthetic group is noncovalently bound haem (protoporphyrin IX) (Fig. 1.12) with an alkaline pyridine haemochrome derivative displaying an α-peak at about 556-558 nm, and excludes haemoglobin and protohaem-containing peroxidases but not cytochrome *o*, a protohaem protein which lacks typical haemochrome properties. The haem can be removed from the protein by treatment with acidified acetone or methyl-ethyl ketone, whereby the apoprotein remains in the soluble phase. Typical mitochondrial and some bacterial cytochromes *b* have ferrohaemochrome spectra with α,

β and γ-peaks at 560-565 nm, 530 nm and 430 nm, respectively (Fig. 1.14). Although cytochromes b have widespread occurrence in bacteria only a few have been isolated, usually with reduced α-bands in the region of 557-562 nm.

Those cytochromes b which are part of the membrane-bound electron transport chains are highly insoluble and have proved refractory to purification without sometimes drastic changes in their physicochemical properties. Other b-type cytochromes appear to be either loosely membrane-bound or localised in a soluble fraction of the cell and are thus more readily isolated. Membrane fragments or purified components thereof containing b-type cytochromes in combination with other enzymes have been isolated, for example formate dehydrogenase and nitrate reductase in *E. coli* and *Wolinella succinogenes*, demonstrating some of the functional associations of these proteins. Despite this functional and spatial diversity the isolated cytochromes b all appear to be typical protohaemochromes and thus have similar absorption spectra except for small differences in the position of the absorption peaks. As with cytochrome c evolutionarily unrelated b-type cytochromes exist according to amino acid sequence data, and fifth and sixth field ligands can be bishistidyl or histidine-methionine (von Jagow & Sebald, 1980).

The best-characterised bacterial cytochromes b are

Fig. 1.14 Spectra of cytochromes b



Redrawn from Weiss & Ziganke, 1978

probably those found in association with the formate dehydrogenase of *E. coli* and *W. succinogenes*, and with the *E. coli* nitrate reductase and *W. succinogenes* fumarate reductase.

Formate dehydrogenase isolated from the cytoplasmic membrane of nitrate-grown *E. coli* is similar to nitrate reductase and consists of three polypeptides: subunit A, a 110 000 d selenoprotein; subunit B of 32 000 d and subunit C of 20 000 d which, by analogy with the nitrate reductase is proposed to be the haemoprotein (Enoch & Laster, 1975). The enzyme also contains Fe-S clusters and contains the same molybdenum cofactor as nitrate reductase. The haem subunit has a midpoint potential of -105 mV at pH 7 (Hackett & Bragg, 1982). Formate dehydrogenase pumps protons across the cytoplasmic membrane at the expense of formate oxidation by menadione or ubiquinone-1, a process which was observed to be sensitive to inhibition by HQNO (Garland *et al.*, 1975; Jones, 1980a)

The formate dehydrogenase of *W. succinogenes* has a different subunit structure; it consists of a 110 000 d molybdoprotein containing nonhaem iron and a 25 000 d cytochrome *b* (Kroger *et al.*, 1979) which is formate-reducible. At -200 mV the redox potential of the cytochrome is lower than that of the cytochrome b_{FDH} of *E. coli* but like *E. coli* electron transfer from formate to quinone is inhibited by alkyl

hydroxyquinoline-N-oxide.

The cytochrome b associated with the nitrate reductase of E. coli (b^{NR}_{556}, +10 mV: Reid & Ingledew, 1979) is part of a 2 :2 :4 complex where the 20 000 d cytochrome b is the γ-subunit. This subunit is essential for quinol oxidation by nitrate, but not enzyme activity with reduced viologen dyes (MacGregor, 1975). Oxidation of reduced cytochrome b^{NR} in the enzyme by nitrate is sensitive to inhibition by HQNO (Crispin et al., 1979).

The fumarate reductase complex of W. succinogenes consists of three polypeptides in a 1 : 1 : 2 stoichiometry (Section 2.2.1). The two 25 000 d subunits are haemoproteins containing protoporphyrin IX and have a midpoint potential of -20 mV at pH 7 (Kroger, 1980). Presence of this subunit in the complex is essential for oxidation of succinate by quinones, and for reduction of fumarate by naphthohydroquinones but not viologen dyes: thus its role is proposed to conduct electrons from menahydroquinone to the (Fe-S centres of the) fumarate reductase complex in vivo.

In addition to cytochrome b^{FDH} and b^{NR}, E. coli contains a number of other b-type cytochromes which can be detected in cells grown under a wide variety of aerobic and anaerobic conditions. Under aerobic conditions with minimal contributions from components of the low aeration respiratory chain, cytochrome o as

well as two to four potentiometrically and spectrally distinguishable b-type cytochromes have been observed (Reid & Ingledew, 1979; Van Wielink et al., 1983). The cytochrome o was solubilised by Hackett & Bragg (1983) as a complex containing cytochromes b_{562} (+196 mV), b_{555} (+129 mV) and traces of b_{556} (-43 mV). Matsushita et al. (1983) purified a cytochrome o-containing complex from an octyl--D-glucopyranoside-solubilised membrane extract of E. coli which contained four proteins on denaturing PAGE gels of 68 000 d, 34 000 d, 21 000 d and 14 000 d. It contained two b-type haems and bound CO, but it was not determined which or both of them bound the ligand. These results were complemented by Kranz & Gennis (1983) who used antisera against cytochrome o to precipitate uniquinol-1 oxidase activity from detergent extracts of E. coli membranes. Their preparations contained four subunits of 51 000 d, 28 500 d, 18 000 d and 12 700 d, and two b-type haems were resolved by 77°K spectroscopy absorbing at 555 nm and 562nm.

Kita et al. (1984; 1984b) purified a cytochrome bo complex from E. coli membranes, which contained two subunits of 55 000 d and 33 000 d. Two b-type haems were present: cytochrome b_{562} (125 mV) and cytochrome o (b_{555}), and 2 mol copper per mol complex. When reconstituted into proteoliposomes the complex pumped protons at the expense of ubiquinol-1 and oxygen, and was inhibited by HQNO. A cytochrome bd complex was

purified from the same membranes (Section 1.3.4) thus providing direct evidence for branching of respiratory sysems (Section 1.2.1) and also for the function of three of the b-type cytochromes found in aerobically-growing E. coli (Reid & Ingledew, 1979; Van Weilink et al., 1983), which are accounted for by the cytochrome bo complex and the cytochrome bd complex. The remaining cytochrome b which has been reported in many studies is the low-potential cytochrome b_{556} which was proposed to mediate electron transfer between two discrete quinone pools in aerobically-grown E. coli (Downie & Cox, 1978; Kita & Anraku, 1981). This cytochrome has been purified as a ubiquinone reductase of 17 500 d with a single haem b prosthetic group and a midpoint potential at pH 7 of about -45 mV (Kita et al., 1978).

Cytochromes o

Cytochromes o are b-type cytochromes which exhibit light reversible CO-binding character (Castor & Chance, 1955; Chance et al., 1953), which can be used to distinguish these cytochromes potentiometrically from multiple b-type cytochromes since carbonmonoxy-cytochromes o exhibit a higher midpoint potential than in the native state. The CO-difference spectra of cytochrome show absorbance peaks at 416,

540, 578 nm and troughs at 432 and 560 nm. Two types of cytochrome o complex have been purified, designated co and bo (Froud & Anthony, 1984; see also previous section) depending upon the prosthetic group of the cytochrome with which they copurify. In addition to haem, cytochromes o may contain copper, such as the cytochrome bo of E. coli, the function of which is probably to serve as a store or buffer of electrons for the four-electron reduction of dioxygen to water.

Few of these complexes have been isolated to date. The E. coli complex has been well-characterised (Kita et al., 1984a; 1984b): the electron donor in vivo is probably ubiquinol-8. Rhodopseudomonas palustris (King & Drews, 1976) contains a co complex, probably a heterodimer of 29 000 d and 22 000 d, and similar complexes have been isolated from Ps. aeruginosa and Methylophilus methylotrophus (King & Drews, 1976; Carver & Jones, 1983). Carbon monoxide binds to both haem types in these cytochromes giving a double trough in CO difference spectra at 550 nm and 560 nm. There is accumulating evidence that the electron donor to the co-type oxidase is of the Class I type which includes horse-heart cytochrome c and cytochrome c_2 from phototrophic bacteria (Pettigrew & Moore, 1986).

1.3.3 Cytochromes c

The haem prosthetic group of c-type cytochromes is

linked covalently to the polypeptide chain (Fig. 1.12) by a pair of cysteine residues following the general pattern Cys-X-Y-Cys-His, with two exceptions. The mitochondrial cytochromes *c* of a small number of protozoa have one of the cysteines replaced by a alanine residue and a single covalent bond attaches the haem group to the polypeptide (Perini *et al.*, 1964; Pettigrew *et al.*, 1975). The tetrahaem cytochromes c_3 from *Desulfovibrio vulgaris* and *D. gigas* have a four-residue spacer separating the cysteines which bind haems 2 and 4 rather than the two-residue spacer found in haems 1 and 3 of the same protein, and all other *c*-type cytochromes (Meyer & Kamen, 1982). The alkaline pyridine absorption spectra of *c*-type cytochromes have an α-peak at 550 nm, while the haem of *b*-type cytochromes have an α-peak at about 560 nm. The protozoal *c*-type cytochromes having only one cysteinyl linkage have an alkaline pyridine spectrum intermediate between the two, at about 553 nm (Perini *et al.*, 1964). Evidence for the identity of a *c*-type cytochrome based on the pyridine haemochrome test is confirmed by acid solvent treatment which denatures the protein and leaves noncovalently attached haems in solution whereas haem *c* is precipitated with the protein.

The *c*-type cytochromes found in the Eukaryotic respiratory and photosynthetic complexes have been fully characterised with respect to structure and function. In contrast, microbial cytochromes *c* exhibit

an extremely wide range of physicochemical properties corresponding to an equally wide range of diverse functions, most of which have not been characterised. This has prompted several different approaches to the systematics of bacterial c-type cytochromes: the scheme described here was derived from structural considerations (Ambler, 1980; Meyer & Kamen, 1982). Three major classes were proposed on the basis of amino acid sequence studies. Between these classes there is little sequence homology, but within classes there is physicochemical and to some extent functional homology, as well as sequence homology.

Class I Cytochromes

Mitochondrial cytochrome c is the representative of cytochromes of Class I, which typically function as electron donors to either the terminal respiratory enzyme or to the oxidised photosynthetic reaction centre. The fifth and sixth field ligands of the haem iron are histidine and methionine, respectively, giving rise to the "695 nm band" in the ferricytochrome spectrum. They tend to have high redox potentials, the variations in which are a reflection of the variation in redox potential of the possible respiratory oxidants. On the basis of sequence and structural data this class has been divided into two main subgroups dependent upon the presence ("large") or absence

("small") of a loop of amino acid residues which closes off the bottom of the haem crevice (Korszun & Salemme, 1977; Almassey & Dickerson, 1978).

The "small" Class I cytochromes are typified by *Pseudomonas* c_{551}, *Chlorobium* c-555, and cytochromes c_4 and c_5. *Pseudomonas* cytochromes c_{551} contain 81-82 residues, have redox potentials of 200-265 mV and probably act as the electron donor to the terminal electron acceptor reductase cytochrome cd_1 and cytochrome *o*. As electron donor to cytochrome cd_1 they are interchangeable with azurin. Its electron donor is probably a bc_1-type complex. The cytochromes c_{555} of *Chlorobium* spp. have 86-99 residues, exhibit redox potentials of 103-154 mV and probably function as electron donors to the oxidised reaction centre, a role which is considered functionally analogous to the oxidation of mitochondrial cytochrome *c* by cytochrome aa_3. The *Chlorobium* c_{555} is probably reduced *in vivo* by inorganic sulphur compounds, in contrast to the reduction of mitochondrial cytochrome *c* by the cytochrome bc_1 complex.

The cytochromes c_4 have relative molecular weight of about 20 000 d and are dihaemoproteins, but sequence and structural evidence strongly suggests that they are the product of duplication of a gene coding for a small Class I cytochrome. Each of the two discrete but very similar domains of 10-12 000 d per Fe have amino acid sequences typical for this subclass (Ambler *et al.*,

1984). The redox potentials of cytochromes $\underline{c}_4$ vary from 335-390 mV, compatible with a proposed function as electron donors to terminal oxidases, but they have not been conclusively characterised with respect to function. They are reactive with Pseudomonas cytochrome $\underline{cd}_1$ in vitro, and cytochrome $\underline{c}_4$ is usually paired with cytochrome $\underline{c}_5$ as electron donor to the terminal oxidase cytochrome o in Azotobacter vinelandii, which also contains a close structural homologue of Pseudomonas $\underline{c}_{551}$. A red coloured fragment of the A. vinelandii membrane has been isolated which contains cytochromes $\underline{c}_4$, $\underline{c}_5$ and o (Bartsch, 1968). Cytochromes $\underline{c}_5$ are enigmatic in that sequence studies reveal ragged N-termini suggesting that they may be a fragment of a larger protein. They contain about 87 residues and exhibit redox potentials of 265-329 mV.

The "large" Class I cytochromes comprise mitochondrial cytochrome c and cytochrome $\underline{c}_2$ found in the purple phototrophic bacteria and at least two nonphototrophic bacteria including Paracoccus denitrificans (Timkovich et al., 1976) and Agrobacterium tumefaciens (Van Beeumen et al., 1980). Mitochondrial cytochrome c conducts electrons from the $\underline{bc}_1$ complex (complex III: ubiquinone-cytochrome c reductase) to cytochromes $\underline{aa}_3$ (complex IV: cytochrome c oxidase). The cytochrome $\underline{c}_2$ of Pa. denitrificans ($\underline{c}_{550}$: +269 mV) functions as the electron donor to cytochrome $\underline{cd}_1$ under denitrifying conditions and is

proposed to have a dual role in aerobic electron transport (Meyer & Kamen, 1982). The phototrophic cytochromes $\underline{c}_2$ function principally as electron donors to photooxidised reaction centre and there is some evidence that cytochromes $\underline{c}_2$ are involved in dark aerobic respiration (Zannoni *et al.*, 1980).

Mitochondrial cytochromes $\underline{c}$ have redox potentials in a limited span from 244-264 mV: the redox potentials of bacterial cytochromes $\underline{c}_2$ vary from 290-390 mV and are pH dependent (Pettigrew & Shejter, 1974). The sequence and structure of the two cytochromes are very similar yet as a result of small differences in surface residues, there is little cross-reactivity with their oxidases. Mitochondrial cytochrome $\underline{c}_2$ will accept electrons from the mitochondrial complex III ($\underline{bc}_1$ complex). The functionally analogous complex III (*bf* complex) from higher plant chloroplasts shows a preference for acidic proteins including its natural oxidant plastocyanin, algal $\underline{c}_{553}$ and *Pseudomonas* $\underline{c}_{551}$ (both "small" Class I cytochromes). The cyanobacterial *bf* complex shows a preference for basic proteins (cytochrome $\underline{c}_{553}$ and plastocyanin, respiratory and photosynthetic carriers respectively for these organisms) such as mitochondrial cytochrome $\underline{c}$. These examples illustrate that for certain cytochromes, a conclusive classification system based on both structural and functional features would be mutually exclusive.

Class II Cytochromes

Two subclasses of cytochrome c are placed within this group (Meyer & Kamen, 1982) and they are very different in terms of absorption spectra and haem coordination: high-spin histidyl-coordinated proteins such as cytochrome c' from phototrophic bacteria, and low-spin his-Fe-met proteins represented by cytochrome c_{556} from Rhodopseudomonas palustris and Agrobacterium tumefaciens. This class of cytochrome is distinguished by the binding of haem near the N-terminal of the protein. The amino acid sequences of c_{556} and c' are very similar, especially that of R. palustris c' which is the reference for the c' family. Cytochrome c_{556} from R. palustris has a relative molecular weight of 13 000 d and a redox potential of 230 mV. The absorption spectrum is low-spin but most of the peaks are shifted to the red and the absorbtivity of the Soret band is unusually high.

Class III Cytochromes

Originally described by Postgate (1956) as the dominant cytochrome component of non-spore-forming sulphate-reducing bacteria, the Class III cytochromes are represented by the cytochrome c_3 of Desulfovibrio vulgaris, which has a molecular weight of

13 000 d, redox potential of -205 mV, and contains four haems. The very low redox potential and the presence of multiple haems are sufficient to place a cytochrome within this group, but they can be distinguished spectrally from other cytochromes by the absence of the 695 nm band, a high $Soret_{(red)}/Soret_{(ox)}$ ratio and a small shoulder on the trailing edge of the reduced Soret band. The relatively weak ligand field of the $\underline{c}_3$ cytochromes is responsible for their binding characteristic with exogenous ligands including carbon monoxide and imidazole (Der Vartanian & LeGall, 1974, 1978). The endogenous fifth and sixth haem ligands are both histidine residues (McDonald et al., 1974; LeGall et al., 1971; Meyer et al., 1979; Higuchi et al., 1981). Meyer & Kamen (1982) identified at least three groups of Class III cytochrome which were spectrally and potentiometrically similar. The first group consisted of the Desulfovibrio cytochromes $\underline{c}_3$, a 14 000 d (approx.) cytochrome found in D. gigas, D. desulfuricans, D. africanus and D. salexigens. Although the cytochromes from these different species share similar molecular weights, redox potentials and absorption spectra, their amino acid compositions are surprisingly different. Physiological roles for the Desulfovibrio cytochrome $\underline{c}_3$ appears to be as oxidant for hydrogenase.

The second group of cytochromes $\underline{c}_3$ are found in photosynthetic organisms: the purple phototrophic

bacteria (Meyer *et al.*, 1971), cyanobacteria (Ho *et al.*, 1979), the green alga *Bryopsis maxima* (Kamimura *et al.*, 1977), the red alga *Porphyridium cruentium* (Krogmann *et al.*, 1977) and the diatom *Navicula pelliculosa* (Yamanaka *et al.*, 1967). The purple bacterial proteins range in size from 15 000 d to 24 000 d and in redox potential from -150 to -260 mV. The algal and purple bacterial proteins appear to be dihaem monomers.

The third group of cytochrome c_3-type proteins are found in coliform bacteria where they apparently function as nitrite reductases. A protein has been purified from *E. coli* (Fujita & Sato, 1963; Fujita, 1966a; 1966b) as a 136 000 d decahaem cytochrome c_{552} with a potential of about -200 mV. Kajie & Anraku (1986) purified a 69 000 d hexahaem cytochrome c_{552} nitrite/hydroxylamine reductase from *E. coli* which was similar in properties and amino acid composition to the 66 000 d hexahaem nitrite reductase from *D. desulfuricans* (Liu *et al.*, 1980; Steenkamp & Peck, 1980).

Several groups of cytochromes do not fit easily into any of these three main classes. The majority of these cytochromes are part of complexes such as cytochrome c_1 of the mitochondrial Complex III, and the cytochrome *c* from *Pseudomonas* cd_1 nitrite reductase. The flavocytochrome *c* of phototrophic bacteria and *Pseudomonas putida*, which function as dehydrogenases

for inorganic sulphur compounds and p-cresol, respectively, do not as yet fit into any of the three main classes but may be allocated in future depending upon amino-acid sequence homology (Meyer & Kamen, 1982).

The covalent attachment of the haem group to the polypeptide chain of c-type cytochromes confers distinct advantages over other cytochromes in terms of purification and characterisation. A common problem with proteins containing noncovalently bound haem is progressive loss of the haem group during purification, leading to underestimates of haem/protein ratios and loss of activity. These problems are not encountered with c-type cytochromes: furthermore, denaturation (for instance by SDS) does not lead to haem loss, resulting in the development of highly sensitive methods for the detection of haem on polyacrylamide gels (Thomas et al., 1976; Wood, 1981). Hence the molecular weight of c-type cytochromes can be determined in crude extracts from cells, permitting comparisons between different cell fractions and cell fractions grown under different conditions. The method is also useful for monitoring the progress of purification of c-type cytochromes. A second reason for the plethora of information available concerning these cytochromes is a reflection of the simplicity of purification; since the majority of c-type cytochromes are either soluble or loosely-bound to the outside of

the cytoplasmic membrane in bacteria (Wood, 1983) the detergent solubilisation step in the purification procedures of other types of cytochrome is not usually necessary.

1.3.4 Cytochromes d

Two structurally and functionally different proteins are included in this group. Cytochromes containing haem d (formerly a_2) are terminal oxidases with distinctive spectral properties arising from the presence of the chlorin haem (Barrett, 1956). Reduced preparations show an absorbance maximum at 628-632 nm, which is shifted 5 nm to the red in the presence of CO. Aeration of cell extracts containing cytochrome d shifts the peak to 648-652 nm. In addition to CO and cyanide, the cytochrome d of E. coli binds oxides of nitrogen with concomitant spectral shift (Ingledew & Poole, 1984). Cytochromes d from several bacteria have been well-characterised, e.g. E. coli (Kita et al., 1984b), Enterobacter aerogenes (Porter et al., 1983), Azotobacter (Kauffman & van Gelder, 1973; Kauffman et al., 1980), Proteus vulgaris (Sweet & Peterson, 1978), Pseudomonas putida (Castor & Chance, 1959) and Photobacterium phosphoreum (Watanabe et al., 1979). The cytochromes d of E. coli, Proteus vulgaris, P. putida and Photobacterium phosphoreum have all been shown to exist as bd complexes (Kita et al., 1984a):

they are often associated with cells growing under conditions of oxygen deprivation and are relatively resistant to inhibition by cyanide, hence the trivial names "high-affinity" or "cyanide resistant" oxidase. The second type of haem d-containing proteins contain haem d_1 and are typified by Pseudomonas cytochrome cd_1 (discussed in Section 1.2.5).

1.4 Summary

The best-characterised systems of bacterial electron transport are probably the formate-fumarate respiratory system of Wolinella succinogenes and the formate-nitrate system of E. coli. Both of these respiratory systems have been purified, characterised, reconstituted into proteoliposomes with high recovery of activities for bioenergetic studies, and topographically and genetically mapped. In both organisms, the simplicity of the systems in terms of number of components, their monophasic (membrane-bound) nature and their composition - a formate dehydrogenase, a quinone and a reductase - all assisted in their elucidation. The majority of bacterial respiratory systems which have been studied contain more protein components and may be biphasic, i.e. consist of periplasmic components linked to membrane-bound components, and are consequently more refractory to analysis. The respiratory reductases for nitrate,

nitrite, fumarate and TMAO of Shewanella putrefaciens are known to be periplasmic, whereas the formate dehydrogenase is known to be membrane-bound. In addition the organism is known to synthesise b- and c-type cytochromes, so the respiratory system of this organism can be considered as biphasic.

In common with pseudomonads, S. putrefaciens might be expected to contain cytochromes c_{551}, c_4, c_5 and possibly azurin when grown on nitrate. By analogy with the Enterobacteriaceae, cells of S. putrefaciens containing an active respiratory formate dehydrogenase would be expected to contain a low potential b-type cytochrome in association with it, and by analogy with many facultatively anaerobic bacteria where extensive respiratory studies have been undertaken, cells of S. putrefaciens grown under anaerobic conditions would be expected to contain respiratory components characteristic of aerobic growth. Due presumably to a constitutive mechanism of synthesis they are invariably observed to persist into anaerobic growth (Hackett & Bragg, 1983).

An emphasis in this study was placed on the cytochromes since of all the respiratory components (Section 1.3) the cytochromes, in particular cytochromes c (Section 1.4.3), are probably the components which are easiest to characterise and therefore provide information about the respiratory system on which to base further analytical approaches.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Chemicals and Materials

Chemicals were obtained from Sigma London Chemical Company Ltd., Poole, and were of the highest grade available. Bacteriological media were obtained from Oxoid Ltd., Basingstoke. Gel media for liquid chromatography were from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden, except calcium hydroxyapatite which was obtained from BioRad Laboratories Ltd., Watford, Herts. Glass chromatography columns were from either Pharmacia or LKB Instruments Ltd., Bromma, Sweden.

2.2 Bacterial Culture

2.2.1 Bacterial Strain

Shewanella putrefaciens (formerly Alteromonas sp. NCMB400) was obtained from the National Collection of Marine Bacteria (NCMB) held at the Torry Research Station, Aberdeen (now NCIMB Ltd.). The organism was maintained at 4°C on slopes of nutrient agar containing 2% NaCl (NANA medium). Two slopes were routinely kept in use: a master slope and a second slope from which samples were taken for growth. At 2-4 monthly intervals the master slope was used for subculture onto a set of two fresh slopes. The new master slope was assessed for purity as in Section 2.2.5.

2.2.2 Growth Media

Growth of S. putrefaciens was carried out using the basal medium of Wood & Baird (1943) consisting of (g l^{-1}): NaCl, 20; K_2HPO_4, 1; $MgSO_4.7H_2O$, 1; peptone, 5; yeast extract, 2; pH 7.2. For microaerobic growth this medium was supplemented to final concentrations of 30 mM sodium lactate and 10 mM of the appropriate electron acceptor.

2.2.3 Growth Conditions

Cultures of S. putrefaciens were incubated at their optimum growth temperature of 20°.

Aerobic and oxygen-limited cultures were obtained by growth in 2 l Erhlenmeyer flasks containing 500 ml medium in a Gallenkamp Orbital Cooled Shaker (INR 250-010J) at 180 rev. min^{-1}. Growth was stopped at A_{660} 0.5 for aerobic cultures and A_{660} 1.5 for oxygen-limited cultures. Microaerobic growth conditions were obtained by static incubation of culture vessels filled completely with medium.

2.2.4 Measurement of Bacterial Growth

Growth of bacterial cultures was monitored by the increase in absorbance at 660 nm in a 1 cm path length glass cuvette using a single beam spectrophotometer.

(Shimadzu U.V. 120-02).

2.2.5 Pure Culture Assessment

Master slopes and cultures were routinely checked for purity by Gram staining and temperature dependence of growth. As a psychrophile, S. putrefaciens is capable of growth on NANA plates at 20°C, but not at 35°C

2.3 Bacterial Cell Fractionation

2.3.1 Harvesting

Cells were harvested at 10 000 g for 10 min (Sorvall RC-5b, GS3 6 x 250ml aluminium angle rotor, 4°C).

2.3.2 Directly-broken Cells

Directly-broken cells were prepared by washing and resuspending the harvested cells in TR buffer (0.1 M K_2HPO_4, 0.1 M NaCl, 1 mM $MgSO_4.7H_2O$, pH 6.5) to 2 mg ml^{-1} protein, then passing the cells once through a French Pressure Cell (Aminco, Silver Spring, Md., U.S.A.) at 27.6 MPa. The translucent extract ("Crude Extract") was centrifuged at 10 000 g for 10min and carefully decanted to separate any particulate

material. The crude extract was separated into the "French Press Supernatant" and "Directly-broken Membranes" by centrifugation at 160 000 g for 1.5 h (MSE Superspeed, 8 x 25 Ti angle rotor, 4°C). The membrane pellet was resuspended by careful hand homogenisation in TR buffer.

By replacing Mg^{2+} with 5 mM EDTA in the TR buffer, the procedure was used to prepare the "EDTA extract" and "EDTA-extracted membranes".

2.3.3 Spheroplast Preparation

Spheroplasts and periplasm were prepared according to the method of Birdsell & Cota Robles (1967) as modified by Easter (1982). Harvested cells were washed in 10mM Tris.HCl pH 8.0 containing 100 mM NaCl and 1 mM $MgSO_4.7H_2O$, then resuspended in 10 mM Tris.HCl pH 8.0 containing 500 mM sucrose and incubated statically at room temperature for 10 min. Lysozyme (30 ug ml^{-1} final concentration) was added and incubation continued for a further 10 min before addition of an equal quantity of 10 mM Tris.HCl pH 8.0 containing EDTA (1 mM).

Formation of spheroplasts was monitored by phase contrast microscopy of wet mount suspension, and took 15-30 minutes for microaerobically grown cells and 30-45 mins for cells grown aerobically. EDTA in the reaction mixture was deactivated by the addition of

$MgSO_4$ to a final concentration of 5 mM before separation of the periplasm from the spheroplasts by centrifugation at 10 000g for 15 min. Turbidity in the periplasm was decreased by a second centrifugation step at 35 000 g for 30 mins: the pellet was added to the spheroplasts which were carefully resuspended in T- buffer (10 mM Tris.HCl, pH 8.4). Where spheroplasts were required intact for enzyme assays they were resuspended in T- buffer containing 0.5 M sucrose and 1 mM $MgSO_4$.

2.4 Spectrophotometric Enzyme Assays

2.4.1 Reductase Assays

Assays for TMAO, fumarate and nitrate reductase were based on the method of Jones & Garland (1977) where the substrate-dependent oxidation of a chemically reduced dye (methyl viologen) was monitored spectrophotometrically.

A Pye Unicam SP6-400 U.V. spectrophotometer connected to a Servoscribe IS RE 541 chart recorder was used to measure the decrease in absorbance at 660 nm caused by oxidation of reduced methyl viologen, $MV^{\cdot +}$ (purple) to MV^{++} (colourless). The assay was performed in 1 cm path length glass cuvettes fitted with Teflon stoppers drilled to accept microsyringe needles up to 0.5 mm diameter. Into a cuvette was placed an aliquot

(5-100 ul) of enzyme and some antibumping granules to facilitate mixing. The cuvette was filled with N_2-sparged (O_2-free N_2; B.O.C. Ltd., Glasgow) TR buffer containing MV^{++} (0.3 mM). The stopper was carefully fitted such that no air bubbles remained in the cuvette, and sufficient freshly prepared sodium dithionite (25 mM in 10 mM NaOH) was added to raise the A_{660} to approximately 0.75. After recording the absorbance at 660 nm for a few seconds to establish the endogenous rate of $MV^{\cdot+}$ oxidation, the reaction was started by addition of substrate to 6.7 mM. Nitrate reductase was assayed in MacLeod buffer (10 mM Tris.HCl, 0.3 M NaCl, 50mM $MgSO_4.7H_2O$, 10 mM KCl, pH 7.0).

Specific activity was expressed as nmol $MV^{\cdot+}$ oxidised min^{-1} (mg protein)$^{-1}$ equivalent to one unit of activity (u) assuming an extinction coefficient of 13 mM^{-1} cm^{-1} for $MV^{\cdot+}$ (Thorneley, 1974).

2.4.2 Dehydrogenase Assays

Formate dehydrogenase : DCPIP was assayed spectrophotometricallly by the method of Ellis (1959) following the PMS-catalysed reduction of DCPIP at 600 nm. Where other electron acceptors were used the assays were identical except that the alternative electon acceptor replaced the PMS/DCPIP couple. The redox dyes used, their absorbance maxima and millimolar

extinction coefficients, are given in Table 2.1. Anaerobic cuvettes (see Section 2.4.1) were filled with N_2-saturated TR buffer, enzyme source (10-100 ul) and redox dye. The endogenous rate was recorded and the reaction was started by the addition of sodium formate pH 7.0 to 2 mM. Specific activity was expressed as nmol redox dye reduced min^{-1} (mg protein)$^{-1}$.

Where electron transport inhibitors were used, they were added to the anaerobic cuvette after the enzyme source and incubated at room temperature for 5 min before addition of the redox dye.

2.5 Pyridine Haemochromes

Cytochromes in crude preparations were converted into their pyridine derivatives by the method of Lanyi (1968). Preparations ($\approx$ 2 ml) were homogenised with acetone (20 ml)/concentrated HCl (20 ul). The white protein precipitate was separated by centrifugation (10 000 g, 20 min, Sorval RC5b/GSA rotor, 4^{o}C). The supernatant containing extractable haem was discarded after spectrophotometric analysis. The precipitate, containing protein and covalently bound haem, was washed four times with 20 ml acid acetone and then dissolved in pyridine/water (1 : 4) containing 0.1 M NaOH.

Purified or partially-purified extracts were converted into pyridine derivatives by mixing with

Table 2.1. Measured wavelengths and extinction coefficients of electron acceptors used for formate dehydrogenase assays.

Redox dye	Measured wavelength (nm)	Extinction coefficient (M^{-1} cm^{-1} x 10^{-3})
DCPIP	600	21[1]
K_3FeCN_6	410	1[2]
Methylene blue	670	8.6[3]
Cytochrome *c*	550	18.7[4]
Flavocytochrome *c*	552	181.2[5]

[1]Lester & De Moss, 1971.

[2]Galante & Hatefi, 1978.

[3]Fischer, 1883.

[4]Hatefi, 1978.

[5]This work.

pyridine/water (1 : 4) containing 0.1 M NaOH at a ratio of 1 : 8 or less, depending on the cytochrome concentration. Difference spectra were recorded using a Pye Unicam SP1800 recording spectrophotometer, after reducing the contents of the sample cuvette with dithionite.

Haem was measured by the method of Bartsch (1971) using a difference millimolar extinction coefficient of 19.1 cm^{-1} for $A_{550_{red}} - A_{500_{ox}}$, or by the method of Wood (1978) using a difference millimolar extinction coefficient of 21.7 cm^{-1} for A_{550} where peak heights were measured relative to a baseline drawn between the troughs at 535 and 565 nm.

2.6 Electronic Absorption Spectroscopy

2.6.1 Chemical Difference Spectra

Reduced minus oxidised difference spectra of whole cells and fractions were recorded using a Pye Unicam SP1800 Recording Spectrophotometer, SP1805 Programmer and Linear Chart Recorder (Philips), unless otherwise stated.

Samples contained within 1 cm path length quartz cuvettes (Hellma 6Q) were placed in the reference and sample positions of the turbid cell holder. The baseline was recorded and then the sample cuvette contents were reduced with ascorbate (10 mM) using PMS

(0.5 mM) as a catalyst. The spectrum corresponding to high potential cytochrome (Em_7 ascorbate/dehydroascorbate = +80 mV) was recorded followed by the fully reduced minus oxidised spectrum using a few crystals of $Na_2S_2O_4$ as reductant. Oxidation of the reference cuvette contents was effected with either $K_3Fe(CN)_6$ (20 mM) for measuring the α and β peaks, or with H_2O_2 (0.03%) if the Soret bands were also to be recorded.

2.6.2 Substrate Reduction

Enzyme-active preparations were carefully pipetted into 1 cm path length cuvettes containing magnetic stirring fleas and slowly stirred for 1 h during OFN surface-sparging to remove O_2. Physiological substrates were tested at various concentrations in order to determine a fixed concentration which would give a convenient rate of reduction, taken as 50% of dithionite reduction within 2-5 min. Substrates were added by injection using a long needle syringe (SGE 50 ul; Belmont Instruments, Glasgow) and the spectrophotometer was set for a 3 min period between scans after recording the endogenous reduction level, generally from 500-600 nm, against a $K_3Fe(CN)_6$-oxidised reference cuvette.

2.6.3 Inhibition of Substrate Reduction and Oxidation

The rate and level of substrate reduction or oxidation in the presence of respiratory inhibitors were obtained by mixing inhibitor (20 ul) with the sample and incubating at room temperature for 5 min before addition of substrate.

2.6.4 TMAO Oxidation of Soluble Cytochromes

Substrate oxidation of cell fractions was carried out by titrating periplasmic c-type cytochromes slowly with dithionite solution until 95% reduction was reached. Reoxidation was then initiated by addition of TMAO to 20 mM. Periplasm freed from particulate matter by centrifugation (160 000 g, 1.5 h) was carefully pipetted into a quartz cuvette containing a few antibumping granules, then placed in the turbid cell position of the SP1800 spectrophotometer. The cuvette was sealed with a drilled Teflon stopper through which syringe needles were inserted for sparging. The cuvette was sparged for 30 min with OFN. The reference cuvette was fully reduced with $Na_2S_2O_4$ to give an inverted absorbance peak at 421 nm. The sample cuvette was titrated to 95% reduction with dithionite solution (20 mM in 10 mM NaOH), monitored by a diminution of the height of the 421 peak. TMAO (10 mM) was then injected into the test cuvette and the absorbance change at 421

nm was followed with time. After full reoxidation the spectrum from 410-440 nm was recorded.

2.6.5 TMAO Oxidation of Membrane-bound Cytochromes

Membranes from cells grown in the presence of TMAO were carefully resuspended to about 2 mg ml^{-1} protein in TR buffer. The procedure described in Section 2.6.4 was repeated, with the addition of aliquots (10 ul) of periplasm (about 1 mg ml^{-1}) to the reaction mixture after the addition of TMAO.

2.6.6 Determination of TMAO-oxidation of High-potential Cytochromes

A 9 : 1 particulate : soluble cytochrome preparation was used as the sample. The procedure given in Section 2.6.4 was used except that the position of the cuvettes was reversed in the spectrophotometer. The fully-reduced minus oxidised difference spectrum was obtained, and used as a reference to titrate a fresh sample to about 95% reduction using dithionite solution. Diminution of the dithionite-titrated peak at 421 nm was then monitored against an H_2O_2-oxidised reference cuvette after the addition of TMAO. After full oxidation was achieved, ascorbate/PMS was added to the test cuvette. An ascorbate/PMS reduced preparation was used as the reference

2.6.7 Carbon Monoxide Spectra

Carbon monoxide (CO) was prepared by the action of concentrated H_2SO_4 on formic acid. Samples were first reduced with a few crystals of Na_2S_2O4, then sparged with CO for 90 s. Spectra were recorded immediately and at 10 min intervals thereafter against a dithionite-reduced reference. The spectra exhibiting the highest peaks were retained for analysis.

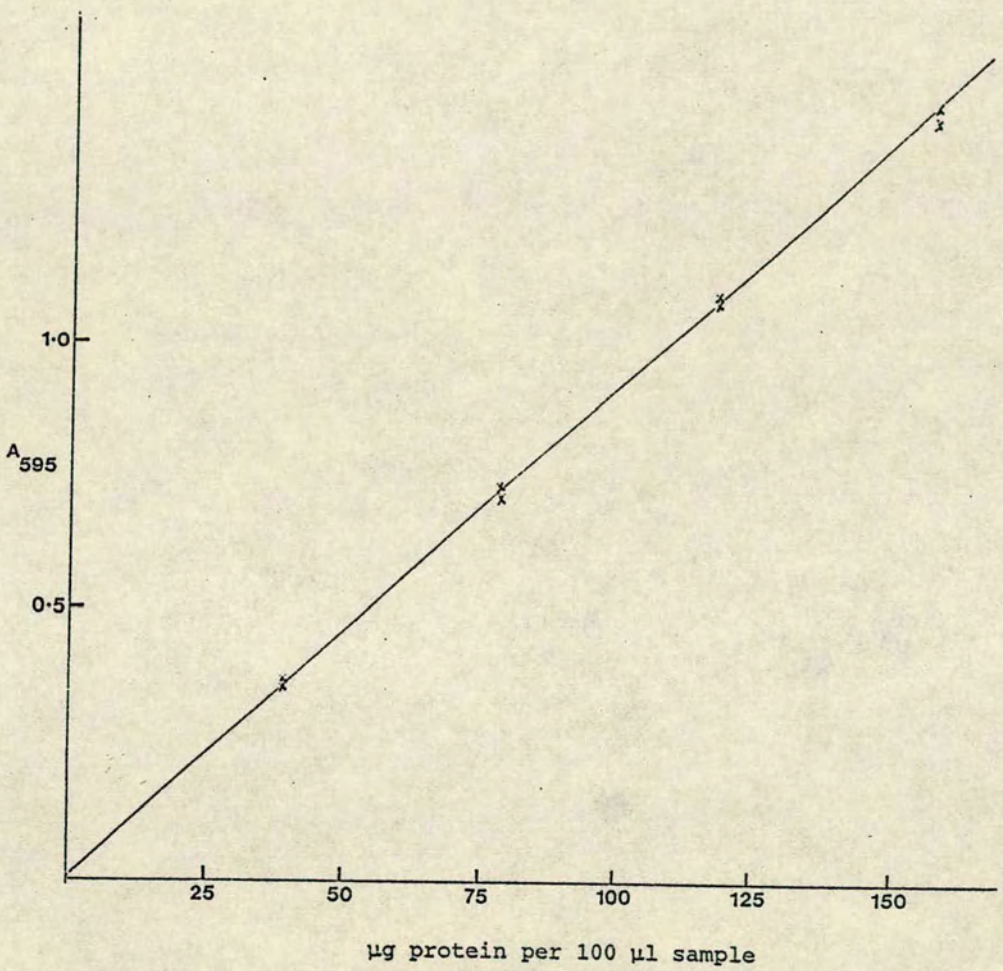
2.6.8 Cyanide Spectra

The oxidised plus CN^- minus oxidised difference spectra of preparations were recorded in 1 cm path length quartz cuvettes after the addition of NaCN (1-50 mM) to the sample cuvette.

2.7 Protein Assays

Protein was assayed by the BioRad dye-binding method using either the macro or the microassay procedure as appropriate (Bradford, 1976). Bovine serum albumin supplied by BioRad in preweighed vials was used as the standard protein except where stated during purification procedures when cytochrome c (horse heart) was used. A typical calibration curve is given in Fig. 2.1. Pure protein preparations were assayed

Fig. 2.1 BioRad calibration curve



(Bovine serum albumin)

for proteins by amino-acid analysis (Section 2.8).

2.8 Amino Acid Analysis

Cytochrome preparation containing approximately 10 nmol haem was freeze-dried in a small Pyrex tube (7.5 cm x 1cm). Reagents were added to give final concentrations of 6 M HCl and 0.05% v/v thioglycollic acid in a volume of 300 ul. The tubes were stored at -20°C for 30 min, then vacuum sealed prior to hydrolysis at 105°C for 20 h or 70 h. The contents were then dried under vacuum and redissolved in 0.2 M sodium citrate pH 2.2 for application to the amino acid analyser.

Amino acid analysis was carried out as described by Gardner (1981) on a Locarte floor model Mark IV amino acid analyser with a 32 cm column of Locarte resin.

2.8.1 Cysteine Determination

Cysteine was determined as cysteic acid after performic acid oxidation of the apocytochrome (Ambler et al., 1969). Cytochrome (1 mg) was taken for dehaeming. The sample was desalted by passage through a Sephadex G25 column (1 x 10cm) equilibrated with 4.5% formic acid at a flow rate of about 10 ml h^{-1}. The yellow-green flavin band which eluted after the

red-brown cytochrome band was retained and the desalted protein was freeze-dried for 2.5 h. The lyophilised protein was dissolved in 0.5 ml of an 8 M urea solution containing HCl (0.1 M) and 6 mg $HgCl_2$. The protein solution was incubated for 6 h at 40°C before desalting into 4.5% formic acid as previously. Fractions were monitored spectrophotometrically at 280 nm for the protein, which was just visible. Apoprotein fractions were pooled and freeze-dried for 1 h.

For performic acid oxidation apoprotein (1 mg) was dissolved in 1 ml H_2O. Performic acid solution was prepared by mixing 0.1 ml H_2O_2 (30% v/v) and 0.9 ml formic acid (98-100%) and incubating in a sealed tube at room temperature for 1 h. Apoprotein solution (0.1 ml) was added to performic acid solution (0.1 ml) in a hydrolysis tube and stored for 1 h at 0°C. The product was freeze-dried for 1 h then hydrolysed for 10 h at 105°C. The hydrolysate was dried under vacuum and redissolved in 0.2 M sodium citrate pH 2.2 for application to the amino acid analyser. The control for the performic acid oxidised preparation consisted of 0.1 ml apoprotein hydrolysed for 20 h at 105°C in the presence of thioglycollic acid and continued as above.

2.8.2 Treatment of Results

The analyser was calibrated by application of 25 nmol of each of the following amino acids: Asp, Thr, Ser, Glu, Pro, Gly, Ala, CysSH, Val, Met, Ile, Leu, Tyr, Phe, His, Lys and Arg. Colour constants of these standard amino acids were calculated from the chart recording and concentration of the amino acids in the samples were obtained. Peak areas were determined manually using peak heights and peak widths at half peak heights.

2.9 Redox Potentiometry

2.9.1 Design and Construction of Apparatus

A number of experimental constraints dictated the structure of the apparatus:

i) The apparatus had to be used in the turbid cell position of the Unicam SP1800 spectrophotometer.

ii) The apparatus had to permit stirring of both cuvettes without interference to the optical paths.

iii) The sample cuvette had to include a manifold to hold the redox electrode firmly.

iv) The spectrophotometer had to be modified to accept the redox electrode projecting from the sample

cuvette without permitting entry of light into the cell compartment, and also to permit simultaneous spectrophotometric recording and reagent addition.

a) Cuvette Assembly (Fig. 2.2)

This assembly, (Pettigrew, personal communication) consisted of a Hellma 21a Pyrex cuvette fused to a Quickfit B19/21 adaptor, sealed with a silicone rubber bung (Esco (Rubber) Ltd., Teddington, Middlesex) which was drilled to accept a Pt-Ag/AgCl combination redox electrode (Russell pH Ltd., Auchtermuchty, Fife, Scotland) with the platinum tip positioned 2 mm above the light path. Two syringe needles were inserted into the bung for gas outlet (also used for reagent addition) and gas inlet, respectively. The gas inlet had a short length of PTFE tubing (1 mm dia) attached which projected down to 2-4 mm above the fluid surface with 4 ml preparation and cell spinbar in place in the cuvette.

b) Stirring Device (Fig. 2.3)

The stirring device was a rigid structure composed of perspex and soft iron sheeting designed on the basis of the turbid cell basket of the SP1800 spectrophotometer. Cell spinbar stirring fleas within the cuvette were driven from below by Eclipse Dutton E3 magnets attached to vertically-mounted electric micromotors (Johnson No. 1, Matchbox Ltd.). Soft iron sheeting (0.5 mm thickness) was used to shield the photomultiplier from the fluctuating magnetic fields

Fig. 2.2 Cuvette assembly

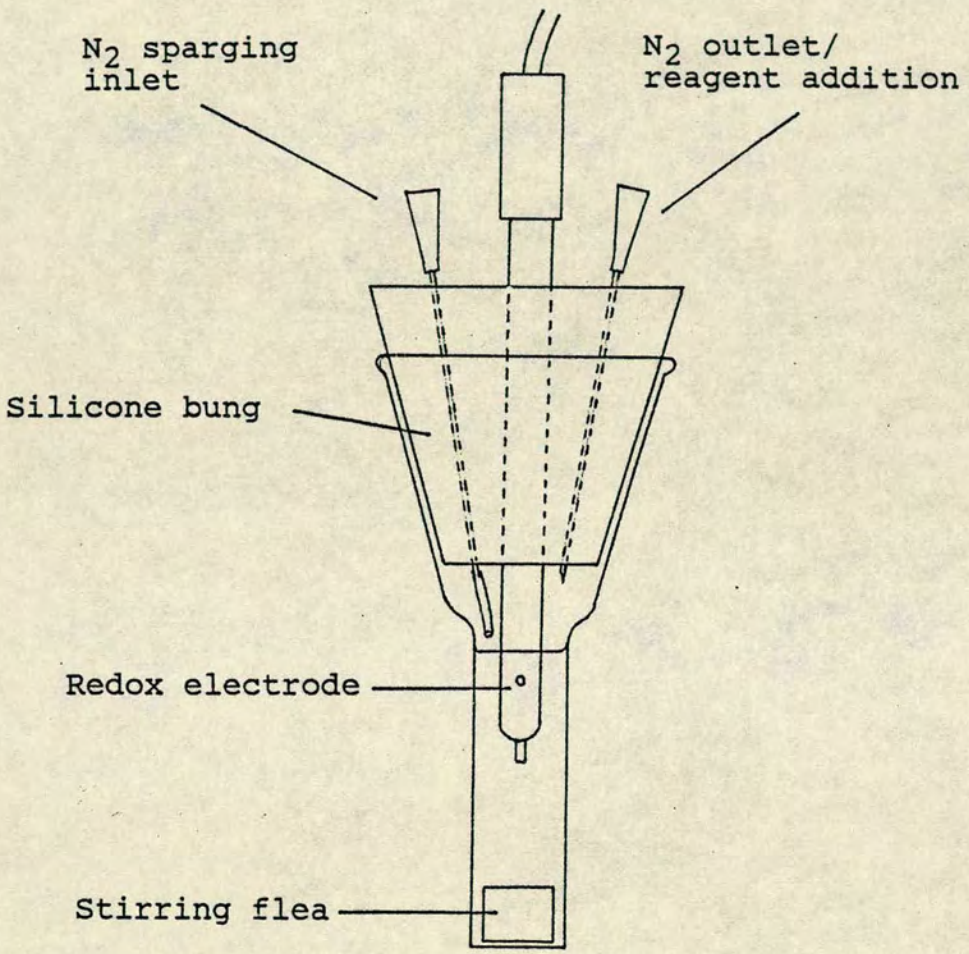
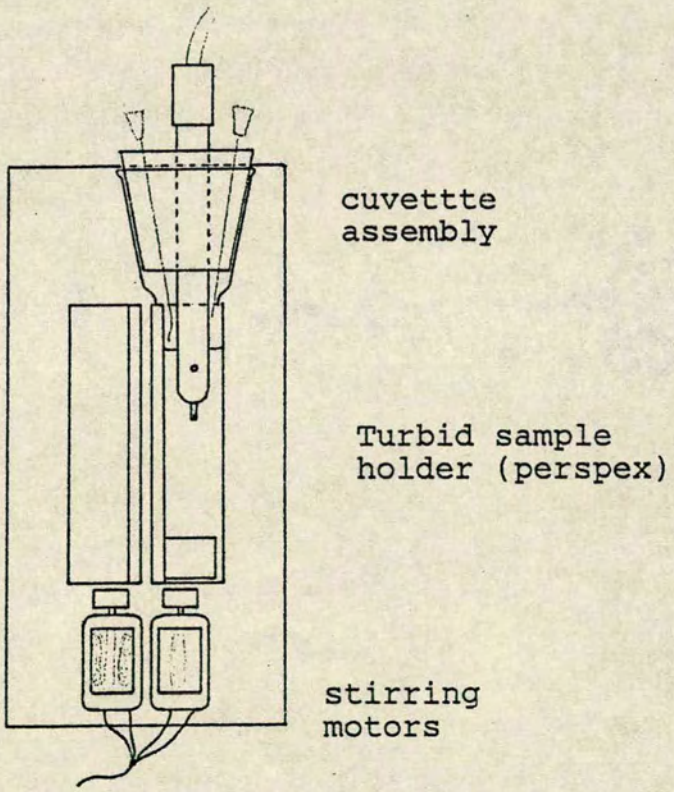


Fig. 2.3 Stirring device



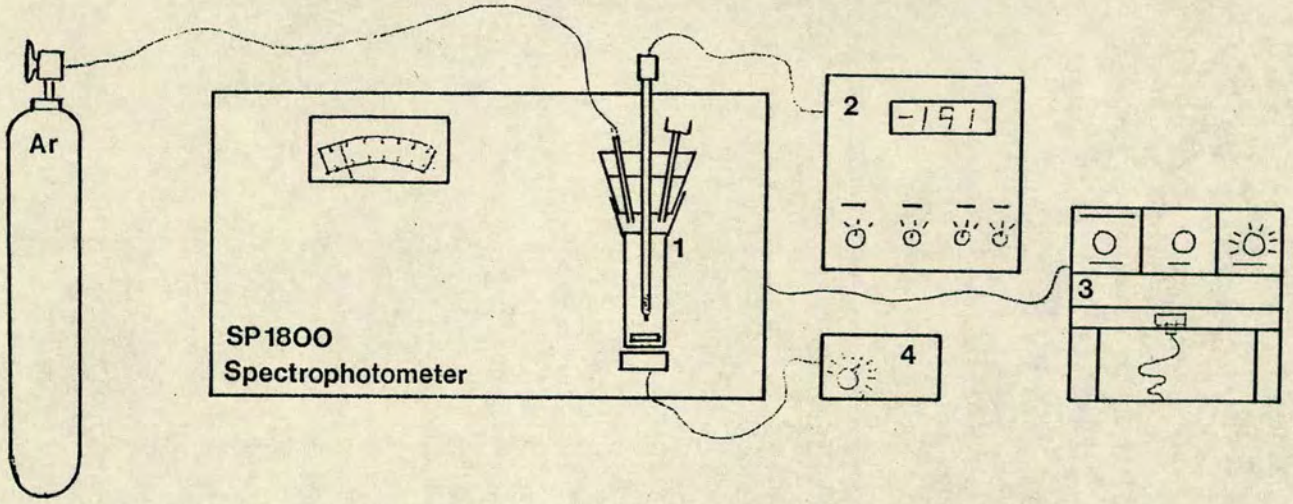
produced by the stirring device. The electric motors were wired for contrarotation for running stability. A substitute sample compartment cover was built for the spectrophotometer in order to accommodate the redox electrode assembly projecting above the surface of the standard cover, and to provide access to instrumental sampling ports. The device was placed over the sample compartment with the normal compartment cover open, and was sealed around the redox electrode housing with black felt cloth. Other equipment is shown in Fig. 2.4.

2.9.2 Electrode Calibration

The combination Pt-Ag/AgCl redox electrode has a potential relative to the hydrogen half-cell under biologically compatible conditions, so the potential of the electrode and any bias of the redox meter must be determined against a solution of known potential. The solution chosen was the "Iron-EDTA" couple with a potential of +112 mV at pH 5.0 (Shwarzenbach & Heller, 1951; Kolthoff & Auerbach, 1952): thus if the electrode/meter combination displays a reading of -88 mV then successive readings during the experiment must have the constant 88 + 112 = 200 mV added to correct the reading to absolute values.

Reagents A, B and C were made up as follows:- A: 2 mM ferric ammonium sulphate/40 mM EDTA tetrasodium

Fig. 2.4 Redox titrimetry apparatus



1. Cuvette/electrode assembly.

2. pH/Eh meter.

3. Pye Unicam AR25 linear chart recorder.

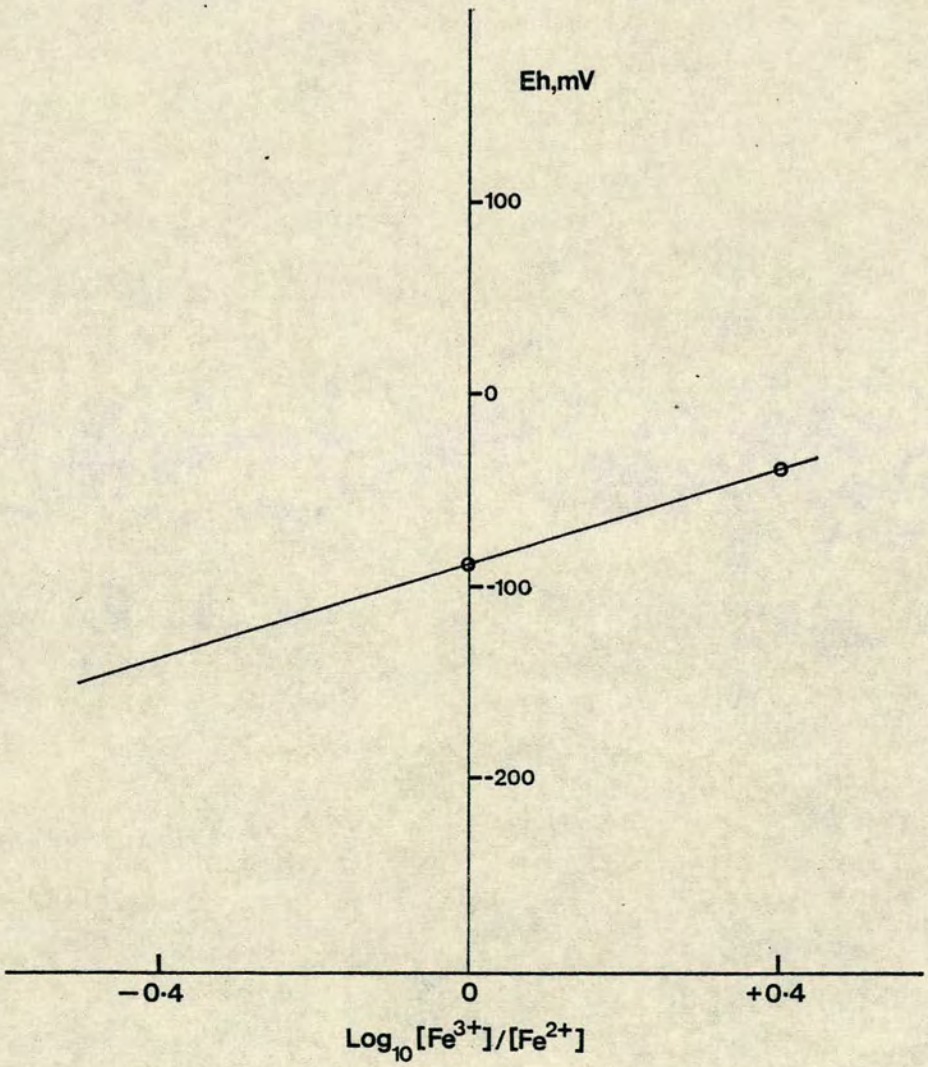
4. Power supply/potentiometer for cuvette stirring device.

salt; B: 100 mM ferrous ammonium sulphate in argon-equilibrated H_2O; and C: 1.0 M sodium acetate buffer, pH 5.0. Reagents A and B were stored in the dark and used within 12 h. Solution A (1.25 ml), Solution C (0.5 ml) and H_2O (3.25 ml) were placed in the redox cuvette and sparged with Ar for 1 h. Solution B (10 µl) was added and the mV reading was measured with an Orion 7091 pH/Eh meter after 2 min equilibration. A further 15 µl solution B was added and the reading was again recorded. The meter readings were plotted against $\log_{10}$ $[Fe^{3+}]/[Fe^{2+}]$ to give a standard Nernst curve where the midpoint potential is the Y-intercept and the slope, typically 59 ± 2 mV, is the change in potential corresponding to a 10-fold change in [oxidised species]/[reduced species] (Fig. 2.5).

2.9.3 Isobestic Point Determination

The reduced minus oxidised spectra of c-type cytochromes consist of a curve with peaks at about 420 nm and 550 nm. The oxidised minus oxidised baseline is a straight line through which the curve passes with the points of intersection being referred to as the isobestic points. These remain constant (wavelength and absorbance) with respect to degree of reduction of the cytochrome. The isobestic points were determined for S. putrefaciens preparations as follows. Samples

Fig. 2.5 Redox titrimetry calibration with Fe/EDTA



(4 ml) were placed in 1 cm path length quartz cuvettes and the oxidised (as prepared) minus oxidised baseline was recorded. The reference cuvette contents were then oxidised fully with $K_3Fe(CN)_6$ (a few crystals) and the spectrum was recorded, overlaid on the top of the baseline. The sampling cuvette contents were then reduced with a few crystals of $Na_2S_2O_4$ and the reduced minus oxidised spectrum was recorded, superimposed over the first two spectra.

2.9.4 Redox Mediators

The redox mediators used for spectrophotometric redox potentiometry have to be colourless in the wavelength region of interest, compatible with the potential of the measured species, and compatible with the hydrophobicity of the measured species (Wilson, 1978). The mediators chosen which satisfied these criteria are given in Table 2.2, and their active ranges are illustrated in Fig. 2.6.

2.9.5 Mathematical Analysis of Titration Data

Analysis of One-component Plots

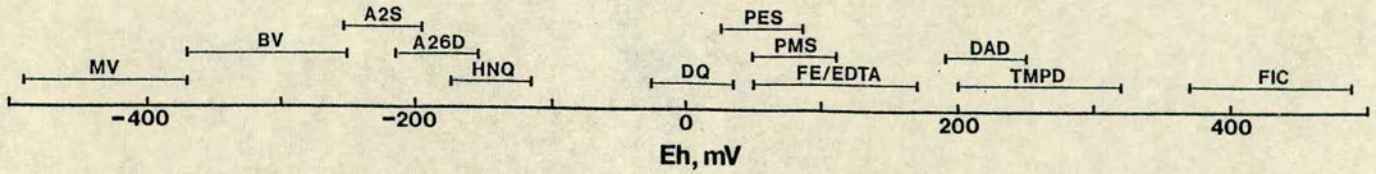
Measured Eh values were converted to absolute Eh values. For each redox point the peak height was converted to the decimal fraction of the peak height at full reduction. Hence the percentage of oxidised

Table 2.2. Mediators used for titration of the cytochromes of *S. putrefaciens*.

Mediator	Em_7 (mV)	n
Methyl viologen (MV)	-430	1
Benzyl viologen (BV)	-311	1
Anthraquinone-2-sulphonate (AZS)	-225	2
Antraquinone-2,6-disulphonate (A26D)	-185	2
2-hydroxy-1,4-naphthoquinone (HNQ)	-145	2
Duroquinone (DQ)	+5	2
Phenazine ethosulpate (PES)	+55	2
Phenazine methosulphate (PMS)	+80	2
Fe/EDTA	+96	1
Diaminodurol (DAD)	+260	2
$K_3Fe(CN)_6$	+430	1

n = number of electrons transferred.

Fig. 2.6 Active ranges of mediators used for redox titrations



cytochrome was determined and $\log_{10}$ [oxidised cytochrome] / [reduced cytochrome] ($\log_{10}$ [ox] / [red]) was calculated.

Two graphs were drawn for each set of points.

a) % reduced vs Eh. The half reduction potential is apparent as the Eh at which the cytochrome is 50% reduced (Fig. 2.7).

b) Eh vs $\log_{10}$ [ox] / [red] (Nernst plot). The half-reduction potential is the point at which the lines intersect the Y-axis (Fig.2.8).

Analysis of Multicomponent Plots

Treatment of the data obtained from multicomponent titration was carried out by extracting the values, relative to individual cytochrome species, from the overall data and treating the values as those obtained for a single species. This was achieved by visual determination of the inflexion points between the optical contributions of the cytochromes, from the Nernst plots. The corresponding value of the x-axis was converted to the antilog giving [ox] / [red] relative to total data, at which for a pair of cytochromes with adjacent redox potentials, one is fully oxidised and the other is fully reduced. The percentage reduction of the component was calculated from the equation -

$$100 - \frac{100Y}{1 + Y}$$

where Y is the antilog derived from the Nernst plot. The corresponding calculated values of Eh and % reduction were inserted into a table of data which was

Fig. 2.7 Redox titration of horse-heart cytochrome c

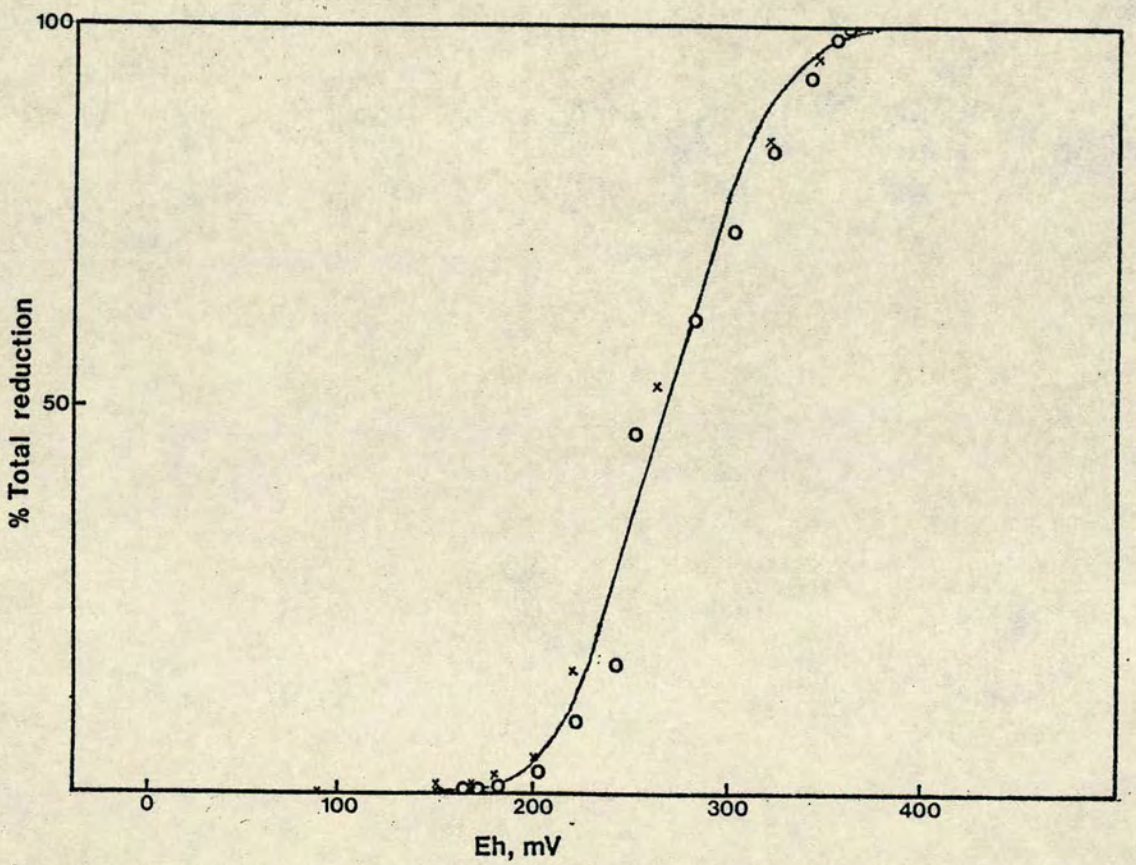
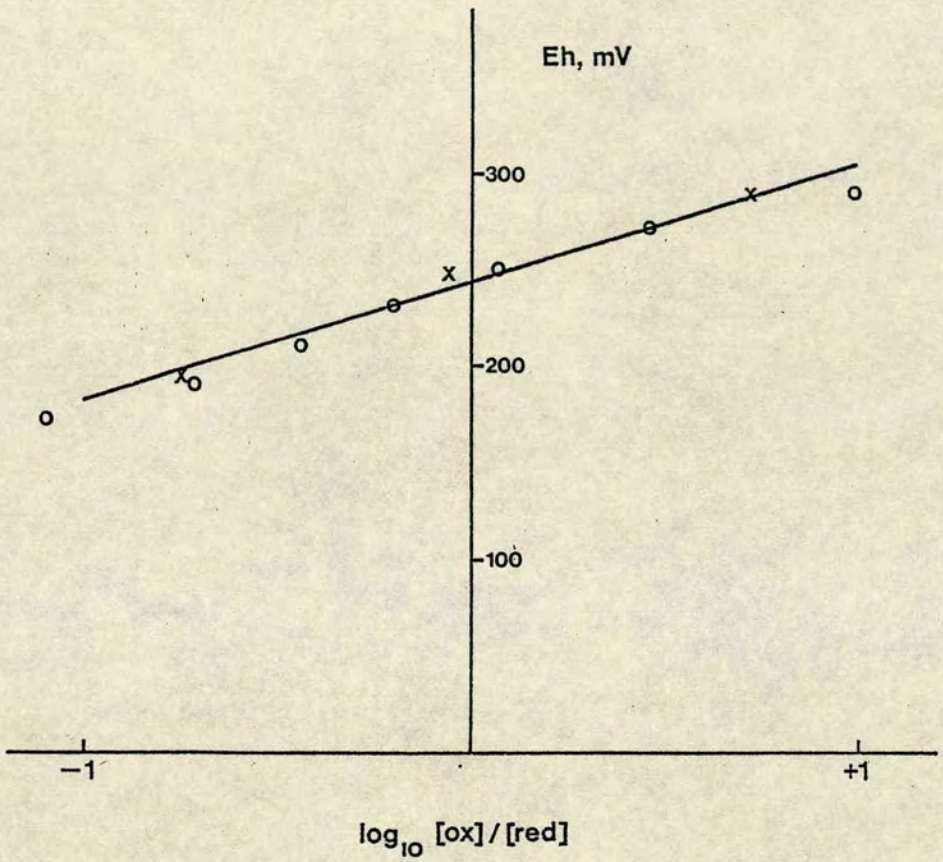


Fig. 2.8 Nernst plot of redox titration of horse-heart cytochrome c



used to generate multicomponent Nernst plots.

2.10 Polyacrylamide Gel Electrophoresis

2.10.1 Electrophoretic Conditions

The method of Laemmli (1970) was used for polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulphate (SDS). Stock acrylamide was prepared from acrylamide (29.2% w/v) and N,N'-methylenebisacrylamide (0.8% w/v) in H_2O and stored in the dark at 4°C. Linear gradient gels (7.5-15%) were used for resolving cell fractions and crude preparations. Purified and partially purified preparations were electrophoresed on 7.5% or 15% gels as appropriate for their molecular weight. Gels were cast as 1 mm slabs with a 20 x 14 cm resolving gel and a 2.5 x 14 cm stacking gel.

Samples for electrophoresis were diluted 1 : 1 with the sample buffer of Thomas et al. (1976) and loaded (5-100 ul) into the sample wells. Electrophoresis was carried out at 10 mA for 1 h followed by 20 mA constant current until the tracking dye reached 1 cm from the base of the gel (3-6 h).

2.10.2 Kenacid Blue Protein Stain

Gels were stained for protein by immersion in

Kenacid Blue (1.25 g) dissolved in methanol (227 ml), glacial acetic acid (46 ml) and water (227 ml). Staining was carried out with gentle agitation for 2-12 h. Gels were destained in methanol (50 ml), glacial acetic acid (75 ml), water (875 ml) and stored in 5% acetic acid.

2.10.3 Cytochrome Stain

Gels were stained for cytochrome by a modification of the peroxidase method of Thomas et al. (1976). 3,3',5,5'-Tetramethylbenzidine (TMBZ) (40 mg) was dissolved in methanol (39 ml) immediately before use, then mixed with 0.25 M sodium acetate pH 5.0 (70 ml). Gels were equilibrated in methanol/sodium acetate 3 : 7 for 5 min then dried and immersed in TMBZ solution in the dark. Staining was carried out for 15 min with agitation every 2 min. H_2O_2 (1 ml of a 30% v/v solution) was added and formation of the blue cytochrome bands was monitored visually under subdued lighting. After full intensity had been reached (30 min), the gels were washed and immersed in isopropanol : 0.25 M sodium acetate pH 5.0 (3 : 7).

2.11 Liquid Chromatography

All procedures were carried out at 4°C. Flow was

provided by peristaltic pumps (Pharmacia P1).

2.11.1 Ion Exchange Chromatography

Anion exchange chromatography was carried out using a 2.6 x 10 cm bed of DEAE Sepharose CL6b equilibrated with 10 mM Tris.HCl pH 8.4 (T- buffer). After sample loading the columns were washed with 2-5 volumes of the same buffer then eluted with an increasing linear gradient of NaCl in the same buffer (1 l). Fractions of 3.75 or 7.5 ml were collected and assayed spectrophotometrically for protein (A_{280}) and haem (A_{410}).

Cation exchange chromatography was carried out exactly as anion exchange except that CM Sepharose CL6b was used as the matrix and the buffer was replaced either by 20 mM sodium acetate pH 5.0 or 10 mM Tris.HCl pH 7.0.

2.11.2 Hyroxyapatite Chromatography

Calcium hydroxyapatite (BioRad, DNA grade) was prepared in T- buffer and packed into a 2.6 x 10cm bed. After loading the sample, the column was washed with one volume of T- buffer. The column was developed with an increasing linear sodium phosphate gradient (0-500 mM, pH 8.4, 500 ml) containing a decreasing linear gradient of T- buffer (10 mM-0 mM, 500 ml).

Fractions (3.75 ml or 7.5 ml) were collected and assayed for protein (A_{280}) and haem (A_{410}).

2.11.3 Hydrophobic Interaction Chromatography

Phenyl sepharose was prepared in T- buffer containing 4.0 M HaCl and packed into a 2.6 x 10 cm bed. Samples were prepared for loading by mixing with an equal volume of T- buffer containing 4.0 M NaCl. After loading, the column was washed with one volume of T-buffer containing 2.0 M NaCl, then eluted with a linear decreasing gradient of NaCl (2.0 M-0 M) in T-buffer.

2.11.4 Molecular Exclusion Chromatography

Sephacryl S-300 superfine was packed into a column with dimensions of 1.6 x 100 cm and equilibrated with T- buffer containing 200 mM NaCl. Samples not exceeding 5 ml volume were loaded, and isocratically eluted with this buffer. Fractions were collected and assayed for protein (A_{280}) and haem (A_{410}). The column was calibrated using horse-heart cytochrome c, 12 400; carbonic anhydrase, 29 000; ovalbumin, 45 000; bovine serum albumin, 67 000; phosphorylase b, 97 000; and β-amylase, 200 000. The void volume Vo was determined using Blue Dextran 2000 (2×10^6). V_e = elution volume of protein and V_t = total column volume.

2.11.5 High Performance Liquid Chromatography

An LKB HPLC system was used, consisting of two HPLC pumps, controller, gradient mixer and Uvicord fixed wavelength (206 nm) U.V. detector. Samples, prepared in 0.1 M sodium phosphate pH 6.8 degassed and filtered through 0.45μm Durapore (Millipore) filters, were filtered through HV4 filter units (Millipore). Samples (100 ul) containing approximately 100 ug protein were injected via an Ultrapac TSK GSWP guard column into a TSK G2000 SW 30 cm gel filtration column using a Rheodyne 7125 injector, and eluted at a flow rate of 0.8 ml min^{-1}. The standards used were ovalbumin, 45 000 d : carbonic anhydrase, 29 000 d; and bovine serum albumin, 67 000 d; 2 mg ml^{-1} protein.

2.12 Flavin Determination

Samples of flavocytochrome containing 71.4 nmol haem in a volume of 700 ul were desalted in a 1 x 10 cm column of Sephadex G25 equilibrated with 4.5% formic acid. Samples (1 ml) were collected and their absorbance was measured at 410 nm and 445 nm. Those samples containing haem were pooled and stored. Samples containing flavin were pooled, and sufficient 2 M potassium phosphate pH 7.0 and H_2O were added to give 10 ml preparations containing 0.1 M potassium phosphate

pH 7.0. Spectra were recorded in the range 300-500 nm against a buffer blank. Flavin was determined from the absorbance at 445 nm assuming a millimolar extinction coefficient for FMN of 12.5 cm^{-1} under these conditions (Whitby, 1953).

CHAPTER 3.

PRELIMINARY STUDIES

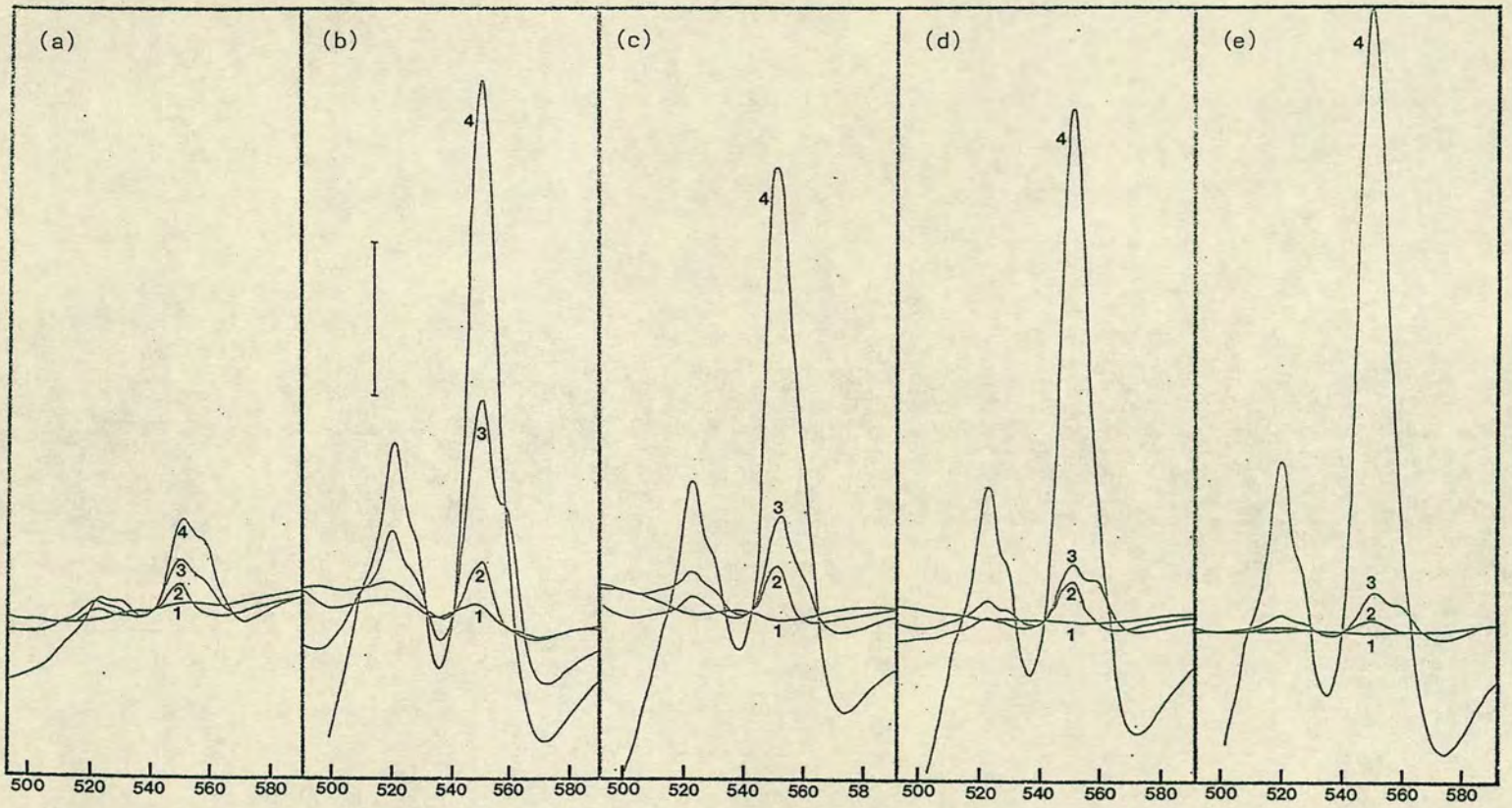
3.1.1 Chemical Reduction/Oxidation

Spectrophotometry of whole cells and extracts using chemical oxidants and reductants was used as a rapid and convenient method for analysing cytochromes. Using this method, terminal oxidases, as well as cytochromes of the b and c-type can be identified, quantitated and localised with respect to the membrane, cytoplasm and periplasm.

High-potential cytochromes

High and low-potential reductants were used to distinguish different cytochromes in S. putrefaciens cell extracts. Spectra are shown of the membrane (Fig. 3.1), periplasmic (Fig. 3.2) and cytoplasmic (Fig. 3.3) fractions. The use of ascorbate as a high-potential reductant revealed the presence of high-potential b and c-type cytochromes (Fig. 3.1). Recording spectra against a $K_3Fe(CN)_6$-oxidised reference consistently revealed a low level of endogenously-reduced cytochrome c which was only clearly resolved in membrane preparations. Cytochrome b_{560} (Fig. 3.4) was only found in the membrane fractions and was fully reduced by ascorbate, indicating that no low-potential cytochrome b was present. A cytochrome c_{552} was also present in these fractions, at higher concentration. The endogenously-reduced c-type cytochromes were distinguished by an α-peak at 552 nm with a small shoulder at about 548 nm. The shape of the spectrum

Fig. 3.1 Chemical reduction of the membrane fraction from aerobic and induced cells

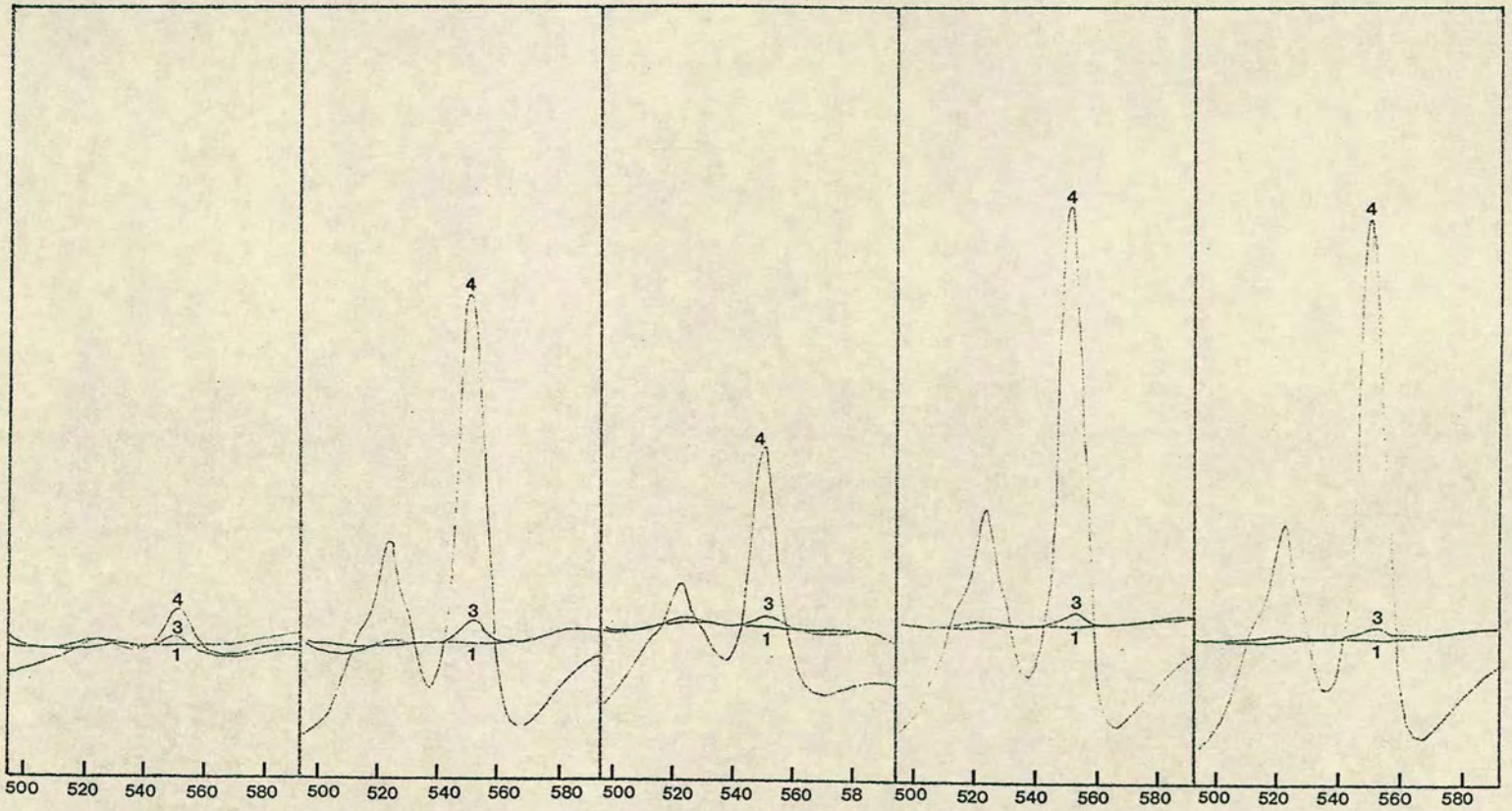


Legends to Figures 3.1, 3.2 and 3.3:
(a) Aerobic (b) Oxygen-limited (c) TMAO grown (d) Fumarate-grown (e) NO_3^--grown

Traces; 1. baseline 2: as prepared vs oxidised 3: ascorbate vs oxidised

4: dithionite vs oxidised

Fig. 3.2 Chemical reduction of the periplasm fraction from aerobic and induced cells

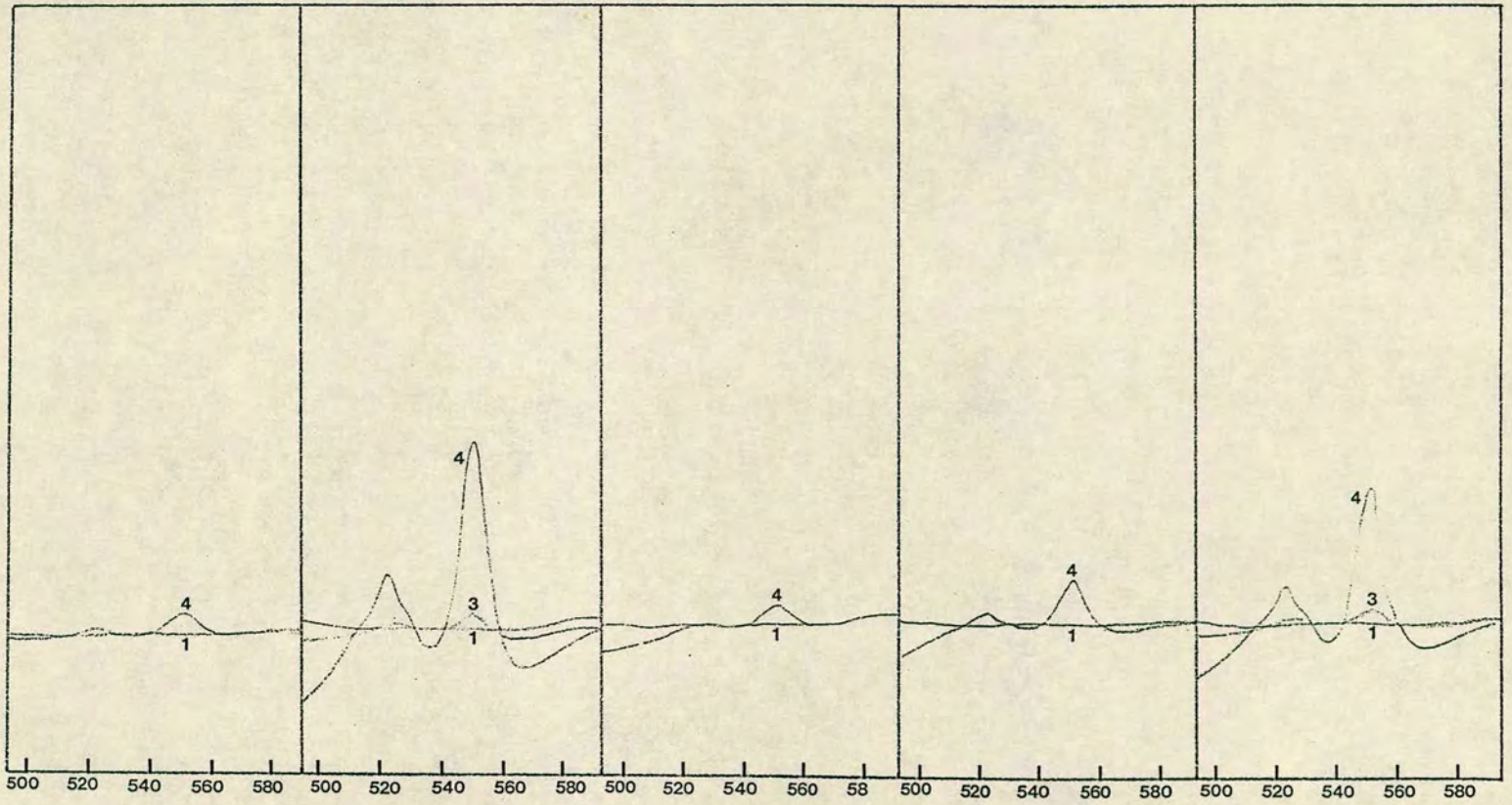


Legends to Figures 3.1, 3.2 and 3.3:
(a) Aerobic (b) Oxygen-limited (c) TMAO grown (d) Fumarate-grown (e) NO_3^--grown

Traces; 1. baseline 2: as prepared vs oxidised 3: ascorbate vs oxidised

4: dithionite vs oxidised

Fig. 3.3 Chemical reduction of the cytoplasmic fraction from aerobic and induced cells

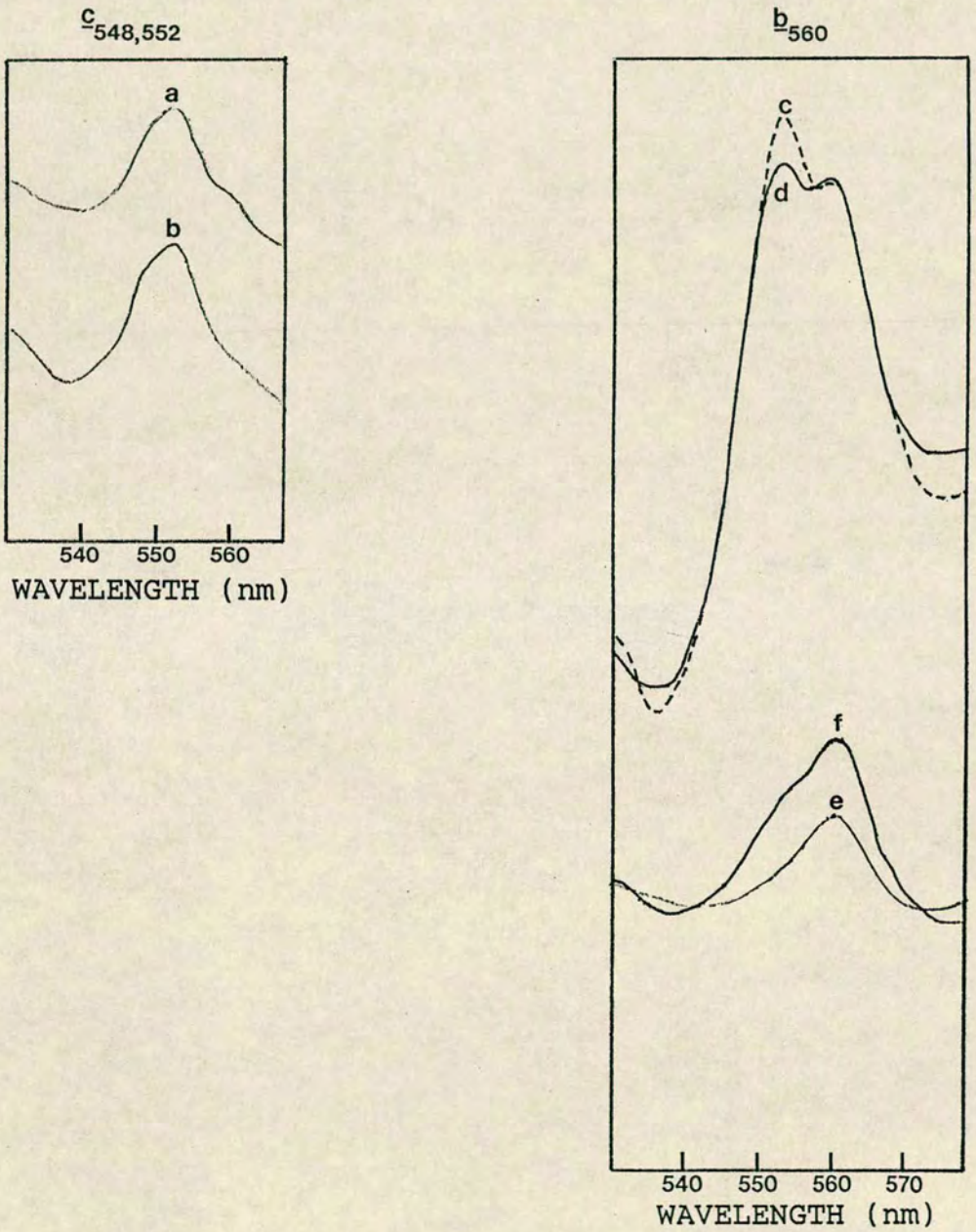


Legends to Figures 3.1, 3.2 and 3.3:
(a) Aerobic (b) Oxygen-limited (c) TMAO grown (d) Fumarate-grown (e) NO_3^--grown

Traces; 1. baseline 2: as prepared vs oxidised 3: ascorbate vs oxidised

4: dithionite vs oxidised

Fig. 3.4 Spectra of cytochromes $\underline{b}_{560}$ and $\underline{c}_{552,548}$ from membranes of S. putrefaciens



a: aerobic membranes, endogenous (as prepared) minus oxidised. b: TMAO membranes, endogenous minus oxidised. c: aerobic membranes, dithionite reduced minus oxidised. d: NADH reduced minus oxidised. e: aerobic membranes, formate reduced minus endogenous, after 3 min. f: after 6 min.

was highly reproducible and may indicate that a single cytochrome species with a split α-peak was present rather than two species with differing α-peaks (Fig. 3.4).

To investigate whether O_2-induced respiratory components were present in fractions from TMAO-grown cells, cells were grown aerobically to a high A_{660} until the haem/protein ratio had greatly increased, suggesting O_2 limitation of growth. These were then compared with TMAO-grown cells . The spectra obtained from the membranes of cells grown under different respiratory conditions are given in Fig. 3.1. The high-potential spectra of membranes from each treatment are qualitatively similar, with an endogenously-reduced cytochrome $\underline{c}_{552,548}$, a high-potential cytochrome $\underline{c}_{552}$ and a high- potential $\underline{b}_{560}$. Another feature shown by Fig. 3.1 and Fig. 3.4 is that aerobically-grown cells contained little (or no) low-potential cytochrome $\underline{c}_{552}$.

In many cases the difference within treatments was the same as the difference between treatments with respect to the relative contribution of each cytochrome species. From the results it may be inferred that the high-potential cytochromes were residual O_2-respiration components but does not exclude involvement with other electron acceptors via branching of electron transport chains.

The soluble cell fractions contained little cytochrome $\underline{b}_{560}$ or $\underline{c}_{552,548}$ (Table 3.1), and when

Table 3.1. Chemical reduction of cell fractions from S. putrefaciens grown under different conditions (nmol haem.mg protein^{-1})

Growth condition	Reductant	Periplasm	Membrane	Cytoplasm
Aerobic	D	0.59	1.23	0.52
	A	0.20	0.68	0.12
O_2-limited	D	5.04	2.25	0.64
	A	0.31	0.93	0.07
TMAO	D	2.33	2.56	0.91
	A	0.10	0.49	0.02
Fumarate	D	9.62	1.57	0.28
	A	0.24	0.17	0.01
Nitrate	D	4.64	2.21	0.73
	A	0.23	0.19	0.03

D = dithionite

A = ascorbate

detected these cytochromes could be removed by centrifugation suggesting that small membrane fragments may have been responsible for the observed spectra.

Low-potential cytochromes

Both periplasmic and cytoplasmic fractions from anaerobic induced and O_2-limited cells contained a small proportion of ascorbate-reducible cytochrome $\underline{c}_{552}$ compared with low-potential cytochrome $\underline{c}_{552}$. The amount of cytochrome in the periplasmic fractions of these cells was very much greater than that found in the membrane or cytoplasmic fractions.

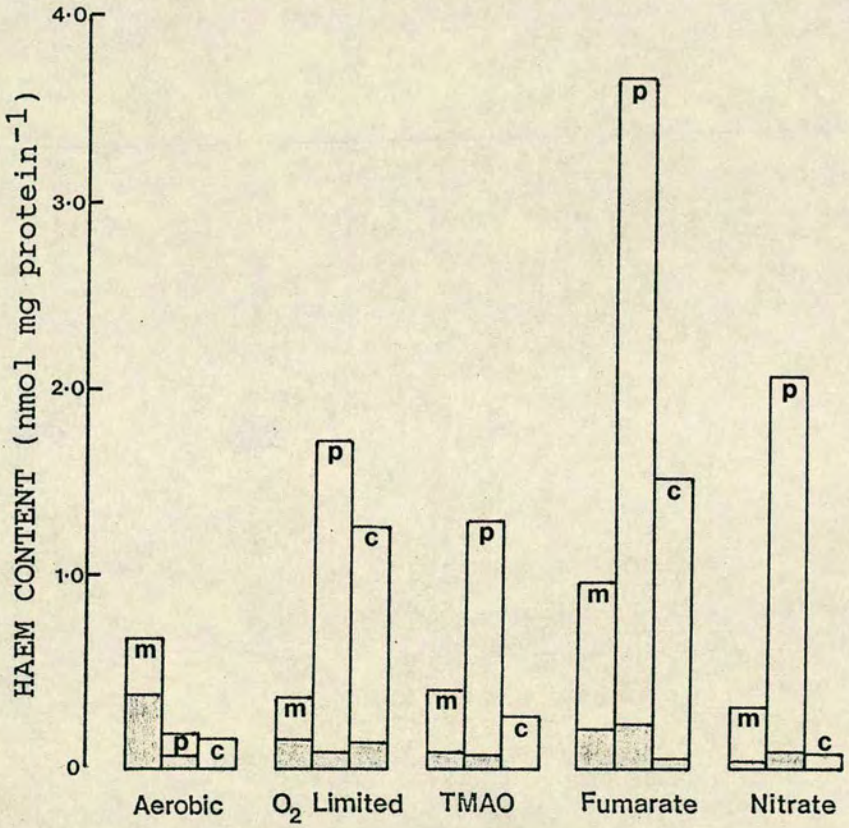
The cytochrome contents of anaerobic induced and O_2-limited cells were very similar. In each case, the low-potential cytochrome $\underline{c}_{552}$ was induced about 20-fold over aerobic cells, the majority of this cytochrome being located in the periplasmic fraction.

Total haem in cell extracts was measured by the pyridine haemochromogen method, and the results are given in Table 3.2. About 60% of the total haem in oxygen-limited and anaerobic induced cells was located in the periplasmic fraction, while the remainder was distributed between the membrane and cytoplasmic fraction. Some variations observed in the membrane and cytoplasmic fractions were due to incomplete sedimentation of particulate matter during centrifugation. The main results from this section are summarised in Fig. 3.5, which gives the relative amounts of low and high potential cytochromes in

Table 3.2. Relative haem content of cell fractions from *S. putrefaciens* grown under different conditions.

Growth condition	Periplasm %	Membrane %	Cytoplasm %	Recovery %	Total haem in whole cells (nmol mg^{-1} protein)
Aerobic	16	63	15	94	1.01
Oxygen limited	56	11	41	108	3.08
TMAO	59	17	11	87	2.21
Fumarate	61	16	26	103	6.01
Nitrate	60	9	22	91	3.45

Fig. 3.5 Relative haem content of cell fractions from S. putrefaciens grown under different respiratory conditions



m = membrane p = periplasm c = cytoplasm

Shaded area = high potential cytochrome

fractions from each growth condition.

Oxidases

Absorption bands of bacterial cytochrome oxidases were rarely detected by difference spectra in any fractions from any of the treatments. Figs. 3.6 and 3.7 show the carbon monoxide difference spectra of aerobic membranes and oxygen-limited membranes, respectively. Both preparations contained CO-reactive species giving rise to troughs at 427 - 433 nm, 552 nm and 615 nm. CO-reactive cytochrome c was present as shown by the trough at 552 nm. This could be attributed to a co-type oxidase complex, or alternatively a discrete CO-binding c-type cytochrome may have been present (see Section 4.1.6 and 5.8): no evidence was found for a CO-binding cytochrome b. The 615 nm band was attributed to the presence of cytochrome d.

3.1.2 Substrate Reduction

Substrate reduction of enzyme-active preparations was first attempted using open unstirred cuvettes and untreated samples. The low level of reduction observed in most experiments was improved by sparging the test cuvette and contents with O_2-free nitrogen (OFN). A standard experimental procedure was adopted using a stirred cell assembly (Section 2.9.2), which usually resulted in immediate reduction on addition of

Fig. 3.6 Carbon monoxide difference spectrum of membranes from aerobically-grown S. putrefaciens

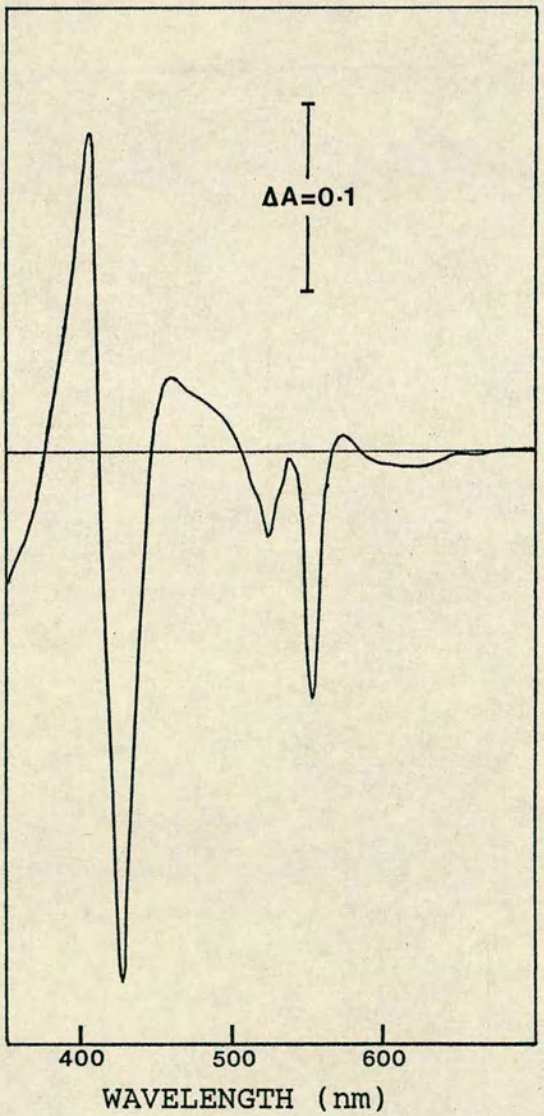
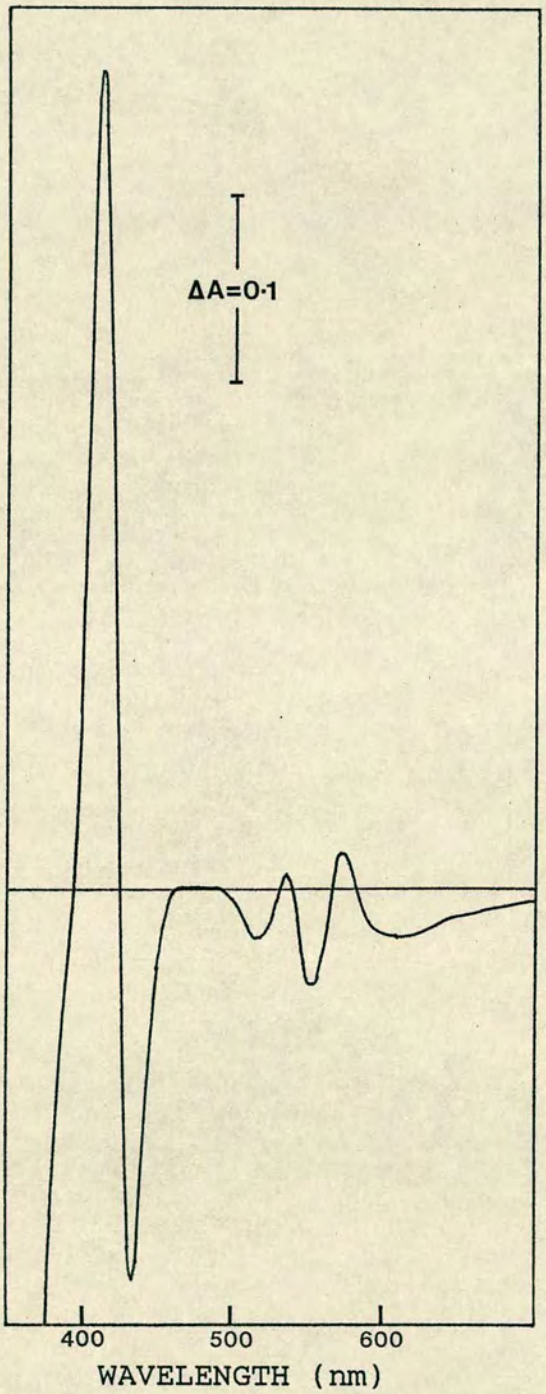


Fig. 3.7 Carbon monoxide difference spectrum of membranes from oxygen-limited S. putrefaciens



substrate. Full reduction was achieved in varying times however, indicating that total exclusion of O_2 was probably not attained. Thus some of the results given in Table 3.3 may have been for steady-state reduction-oxidation of the cytochromes rather than for the highest level of reduction which could be achieved with a substrate.

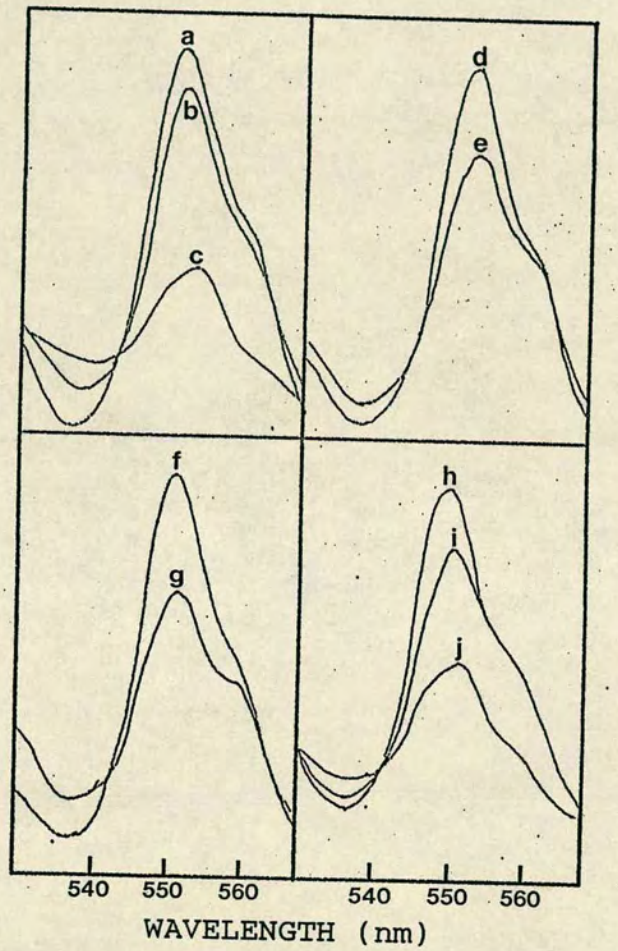
The table shows that NADH (1-5 mM) and formate (14.3 mM) reduced most of the cytochromes in membranes from all growth conditions. Formate tended to achieve a higher level of reduction of cytochromes than NADH, however it was present at a higher concentration than NADH because lower concentrations tended to give a rather long lag phase. In aerobic membranes lactate (10 mM) was as efficient as NADH and formate at reducing the cytochromes, while succinate (10 mM) was noticeably slower although the level of reduction attained after 1 h was the same as that with NADH when preparations with a low proportion of low-potential $\underline{c}_{552}$ were used (Fig. 3.8). In TMAO-grown membranes, lactate (10 mM) was rather slow at reducing the cytochrome complement. Succinate (10 mM) was also rather slow and as expected would only reduce the high-potential components (Fig.3.9).

In order to confirm that formate, via membrane-bound components, could reduce the periplasmic low-potential $\underline{c}$-type cytochromes found in TMAO-induced cells, a mixture of membrane and periplasm (10:90 by

Table 3.3. Relative reducibility of c-type cytochrome in membrane fractions from S. putrefaciens

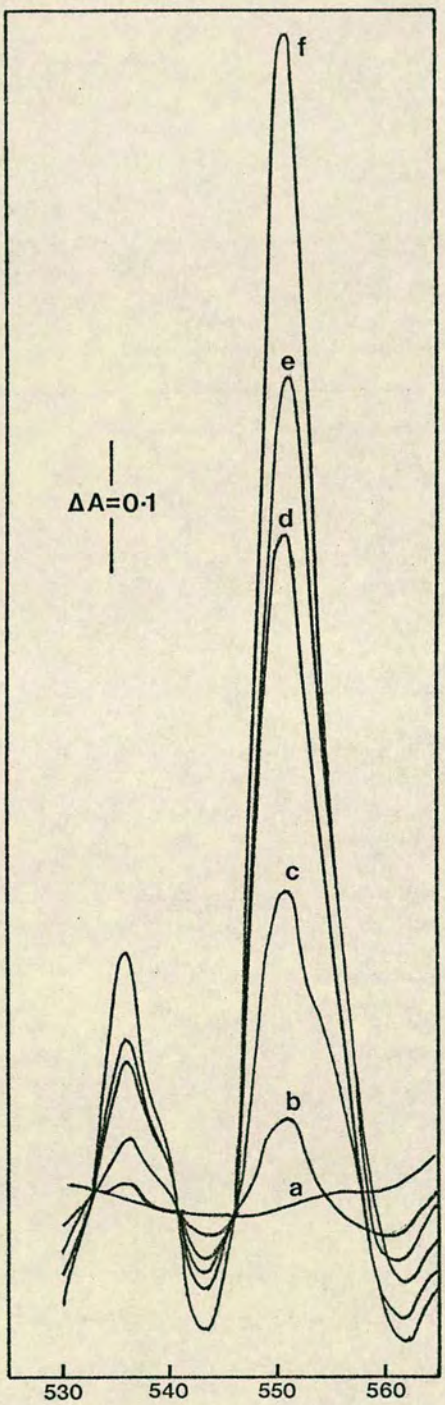
Growth condition	Dithionite	Ascorbate	NADH (1 mM)	Formate (14.4 mM)	Endogenous	Succinate	Lactate
Aerobic	100	55-90	60-70	80-90	20-30	60-70	70-80
O_2-limited	100	20-40	70-80	60-70			
TMAO	100	15-20	70-80	60-100	5-10	20-30	50-60
Fumarate	100	10-20	70-80	80-90			
Nitrate	100	5-10	80-90	80-90			

Fig. 3.8 Substrate reduction of membrane-bound cytochromes from aerobically-grown S. putrefaciens



Reduced minus oxidised spectra with the following reductants: traces a, d, f, h: dithionite. c and j: endogenous reductant. b: NADH. e: lactate. g: succinate. i: formate.

Fig. 3.9 Substrate reduction of membrane-bound cytochromes from TMAO-grown S. putrefaciens



Reduced minus oxidised spectra with the following reductants: a: baseline. b: endogenous reductant. c: succinate. d: lactate. e: formate. f: dithionite.

A_{552}) was prepared anaerobically as for membrane preparations. This ratio provided adequate particulate formate dehydrogenase to permit reduction of the periplasmic cytochromes $\underline{c}_{552}$, while maintaining a sufficiently low level of membrane bound cytochrome to facilitate analysis of the periplasmic cytochromes. Reduction of the preparation with formate (14.3 mM) was rapid, within a few minutes, and subsequent addition of TMAO (14.3 mM) caused complete reoxidation of the low-potential cytochromes (Fig. 3.10).

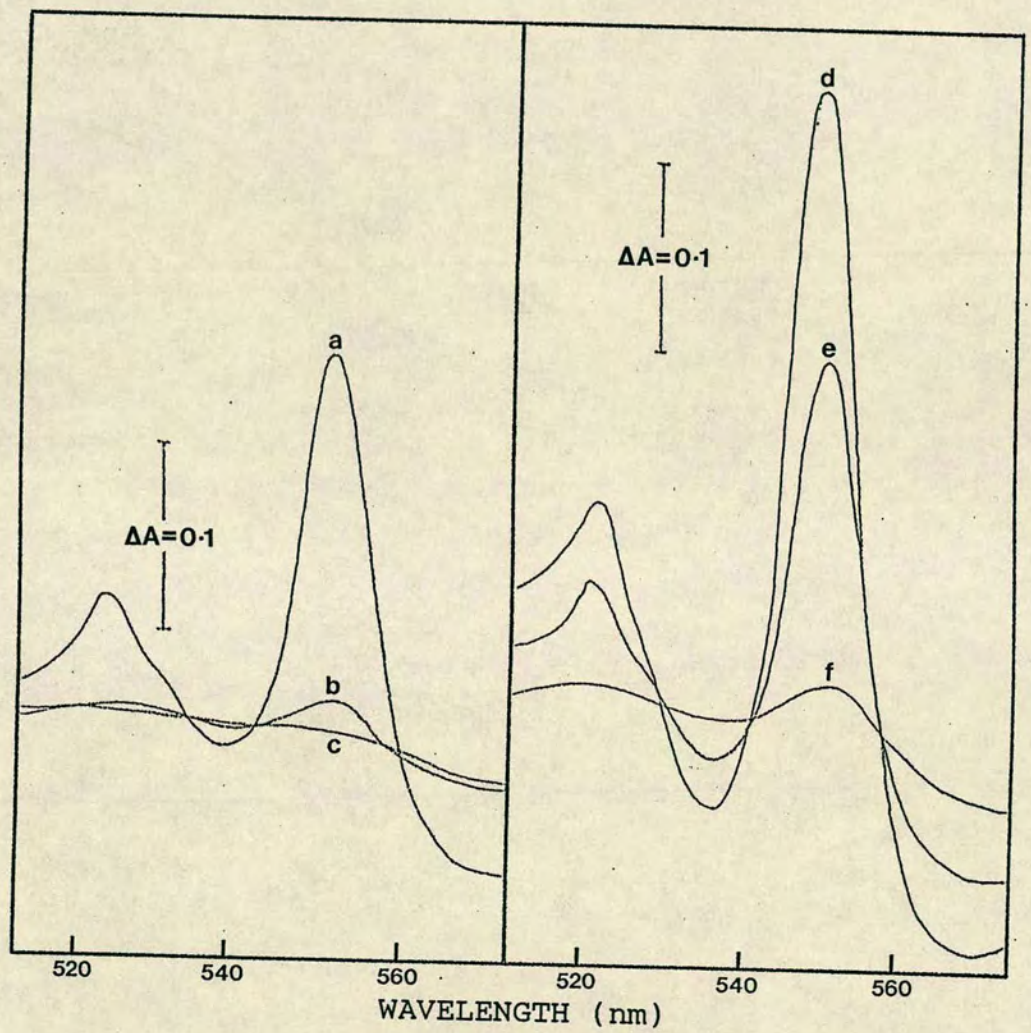
3.1.3 Inhibition of Substrate Reduction

Aerobic Membranes

Substrate reduction of the cytochromes of aerobically-grown membranes was inhibited by HQNO, PCMBS and Antimycin A. NADH (1 mM) reduced 80% of the total cytochromes within 5 min but preincubation of samples with HQNO (50 μM) completely prevented reduction of the cytochromes by 1 mM NADH. Increasing the concentration of NADH to 10 mM resulted in a very slow rate of reduction of cytochromes $\underline{c}_{552}$ and $\underline{b}_{560}$; further increase of the concentration of NADH to 20 mM resulted in 80% reduction of total cytochromes within 1 h. The reduction of cytochromes $\underline{c}_{552}$ and $\underline{b}_{560}$ by NADH was apparently inhibited to the same extent (Fig. 3.11).

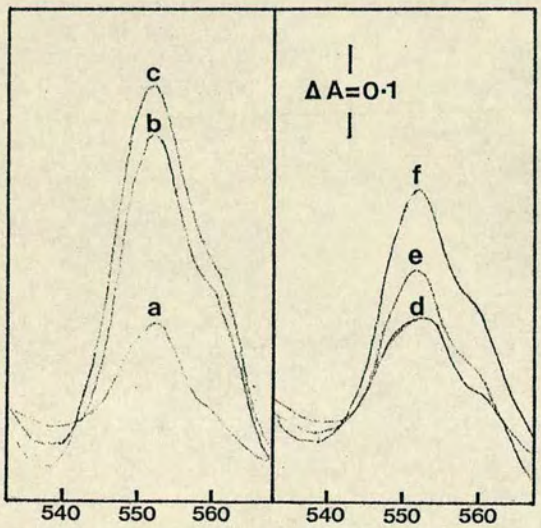
Reduction of aerobic membrane-bound cytochromes by

Fig. 3.10 Formate reduction and TMAO oxidation of the periplasmic fraction from TMAO-grown S. putrefaciens



c: baseline. b: 350 µl membranes added. a and e: formate reduced minus oxidised. f: TMAO reoxidised minus oxidised. d: dithionite reduced minus oxidised.

Fig. 3.11 Effect of HQNO on the reduction by NADH of membrane-bound cytochromes from aerobically-grown S. putrefaciens



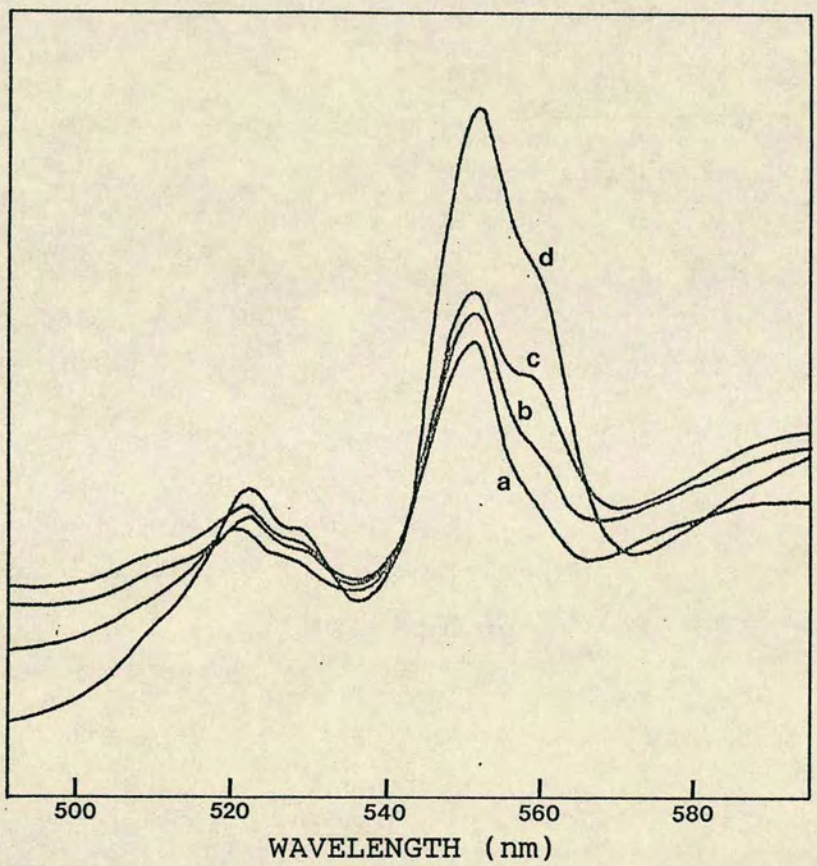
Traces; a: endogenous reduction b: NADH reduction control (2 min) c: dithionite reduction control d: NADH after 5 min in presence of HQNO e: as d, after 10 min f: as d, after 30 min

formate was also sensitive to inhibitors. Samples were preincubated with PCMBS (0.57 mM), HQNO (0.5 mM) or Antimycin A (0.5 mM) before addition of formate, and the level of reduction achieved and the rate of reduction were compared with those of a control sample preincubated for 5 min in the absence of inhibitors. The results indicated that PCMBS, HQNO and Antimycin A were all effective as inhibitors, but that the effects were somewhat different. Formate (14.3 mM) reduced 60-70% of total cytochromes within 5 min in the control. HQNO and Antimycin A caused a total inhibition of reduction of all cytochromes. This suggested that they were inhibiting a component, possibly the formate dehydrogenase itself, prior to the cytochromes in the electron transport sequence. PCMBS in contrast caused an initial complete inhibition of reduction which was relieved with time. Reduction of $\underline{b}_{560}$ was visible after 5-10 min, followed by reduction of cytochrome $\underline{c}_{552}$. Full (60-70%) reduction of membranes from aerobically-grown cells took about 1 h in the presence of PCMBS (Fig. 3.12).

TMAO-grown Cell Fractions

The reduction by formate of membrane-bound cytochromes from TMAO-grown cells was apparently less sensitive to inhibition than in aerobically-grown membranes. Formate (14.3 mM) reduced 70-100% of membrane-bound cytochromes in 10-30 min. When preincubated for 5 min with either HQNO or Antimycin A,

Fig. 3.12 Effect of PCMBS on the reduction by NADH of membrane-bound cytochromes from aerobically-grown S. putrefaciens

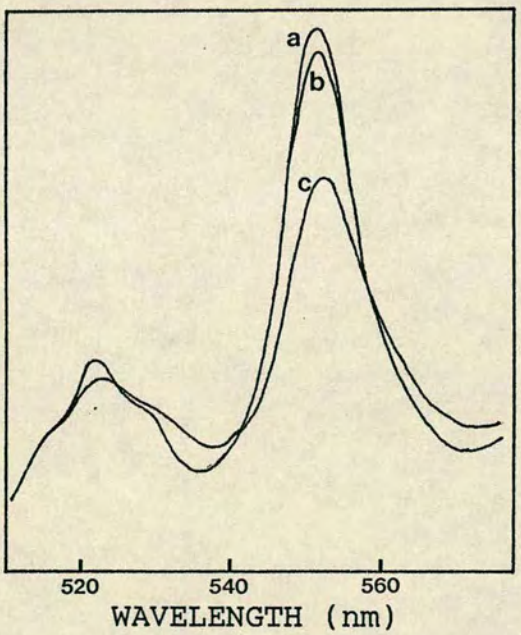


a: endogenous reduction. b: formate (14.3 mM) and PCMBS (0·57 mM) added to test cuvette. c: after 5 min. d: after 10 min.

preparations were reduced by formate to a lesser extent (50-70%) and at a lower rate (2 h) (Fig. 3.13). No difference was observed in the rate or level of reduction between b-type cytochromes and c-type cytochromes, indicating that as in aerobic membranes the site of inhibition of HQNO and Antimycin A was prior to the cytochromes in the electron transport chain.

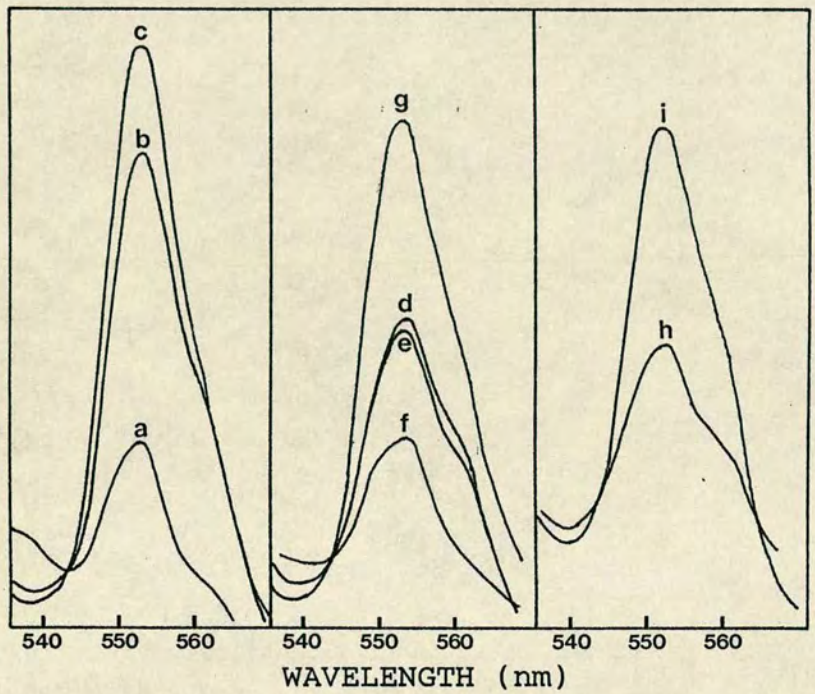
A variation upon this experiment was carried out using a mixture of membranes and periplasm (97 : 3 by protein content) so that steady-state reduction-oxidation of the cytochromes by formate and TMAO could be observed. Formate (14.3 mM) was added 10 min after HQNO (50 uM) and did not reduce the low-potential cytochromes: high-potential cytochromes were present which were already reduced by endogenous substrate. Addition of TMAO (14.3 mM) had no apparent effect. Raising the concentration of formate to 28.6 mM partially overcame the inhibition and cytochromes c_{552} became reduced. Increasing the HQNO concentration to 100 uM resulted in a decrease of the level of reduction of cytochromes c_{552}, and the endogenous level of reduction was restored. Further increase in the concentration of formate again appeared to relieve inhibition: addition of TMAO restored the level of reduction of the cytochromes to that before formate addition (endogenous level) (Fig. 3.14). Exactly the same results were obtained with Antimycin A. These

Fig. 3.13 Effect of HQNO on the reduction by formate of membranes from TMAO-grown S. putrefaciens



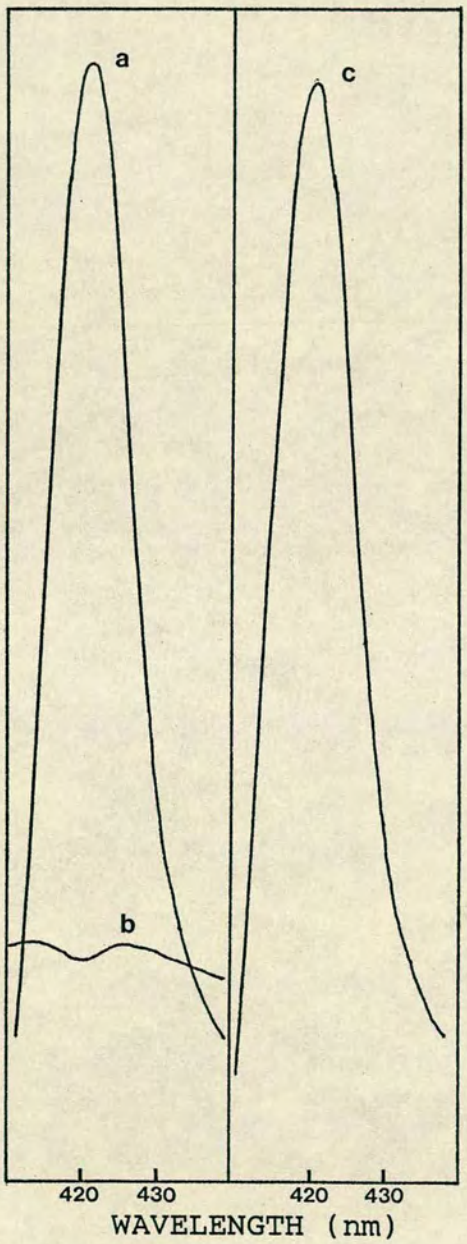
Traces; a: dithionite reduced minus oxidised b: formate reduced minus oxidised c: formate reduced minus oxidised, in the presence of HQNO (0.5 mM)

Fig. 3.14 Effect of HQNO on the reduction by formate and oxidation by TMAO of membrane-bound cytochromes from TMAO-grown S. putrefaciens



Traces; a: as prepared minus oxidised b: formate (14.3 mM) reduced (control) minus oxidised c: dithionite reduced minus oxidised d: formate (14.3 mM) reduced minus oxidised, in the presence of HQNO (50 µM) e: as d, with 2.9 mM TMAO f: as e, with 100 µl periplasm g: as e, with 28.6 mM formate h: as g, with 100 µM HQNO i: as h, with 43 mM formate

Fig. 3.15 Oxidation by TMAO of cytochromes in the periplasmic fraction of TMAO-grown S. putrefaciens



Traces; a: solid dithionite reduced minus oxidised
b: solid dithionite reduced minus dithionite titrated
c: solid dithionite minus TMAO reoxidised

These results indicated that a single site of inhibition by HQNO and Antimycin A was present between formate and TMAO, and that it preceded the cytochromes in the electron transport chain. No oxidant-induced reduction of cytochrome b was observed as in mitochondria and Paracoccus denitrificans (Gabellini et al., 1982).

3.1.4 Substrate Oxidation

Demonstration of substrate reduction of cytochromes in bacterial cells was more easily achieved than a similar demonstration of substrate oxidation since in the latter case the cytochromes first had to be reduced, and then oxidised. Experiments on cytochrome oxidation by TMAO often resulted in steady-state reduction-oxidation of the cytochromes until reductant became limiting.

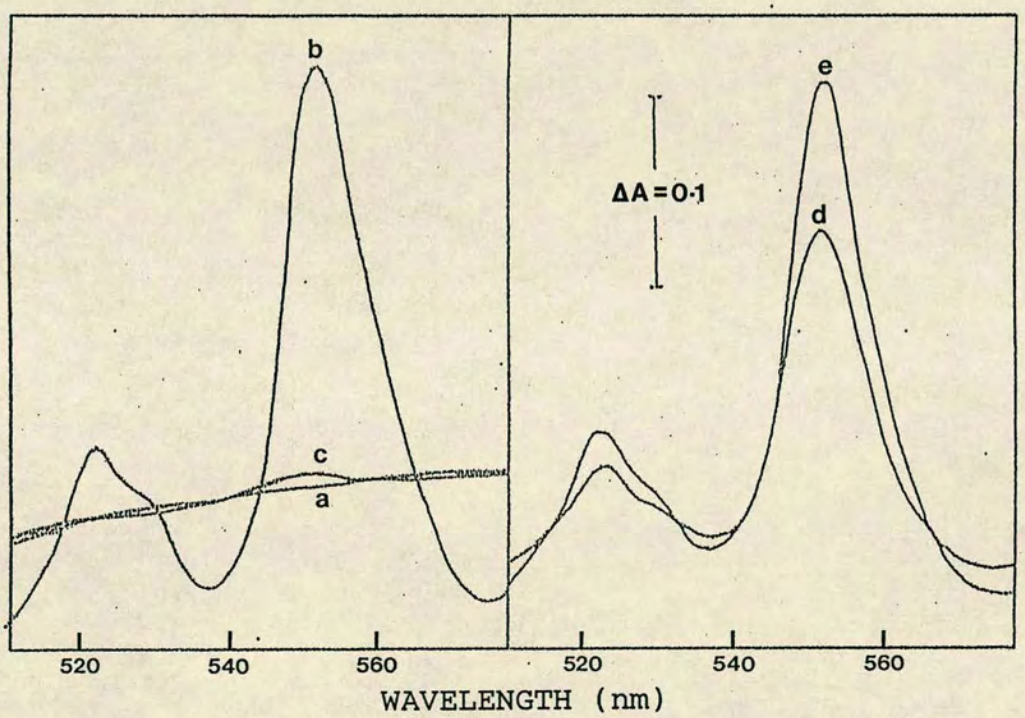
The problem was overcome in this study by using pulses of dithionite solution to reduce preparations to about 95% of full reduction, ensuring no excess of reductant to give rise to steady-state conditions. In practice the quantity of dithionite used did appear sometimes to give rise to a steady-state but the time periods involved were typically less than 2-3 min.

In order to assess the involvement of low-potential cytochromes in TMAO respiration two types of experiment were conducted. The first type (Fig. 3.15) used periplasm as a source of cytochrome c and

was designed to demonstrate the involvement of soluble cytochromes. The second type of experiment (Fig. 3.16) used membranes to demonstrate the involvement of membrane-bound cytochromes in TMAO respiration. In this type of experiment, a small aliquot of periplasm representing not more than 10% of the absorbance at 552 nm (fully-reduced) was mixed with the membrane to provide TMAO reductase and any other necessary components (Section 2.6.4).

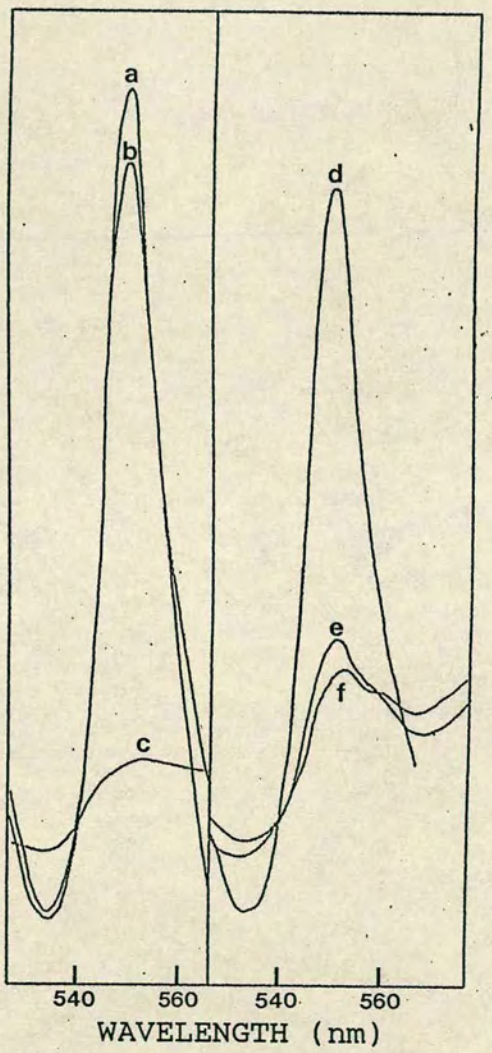
The results indicated that all the low-potential c-type cytochromes in TMAO-induced membrane and periplasm fractions were TMAO-oxidisable. By reversing the cuvettes and changing from a dithionite-reduced minus (dithionite-titrated then TMAO-oxidised) spectrum, to a (dithionite-titrated then TMAO-oxidised) minus K_3FeCN_6-oxidised spectrum, it was possible to show that the residual cytochromes which were not oxidised by TMAO were equivalent to the small complement of ascorbate-reducible cytochromes (Fig. 3.17). Fig. 3.18 shows the results for a similar experiment where the reduced minus oxidised difference Soret band (420 nm) was used to monitor TMAO reoxidation of dithionite-reduced periplasm. All of the low-potential cytochromes were reoxidised by TMAO (14.3 mM) with 3 min. Cytochrome $c_{552,548}$ was not observed since it was masked by c_{552}.

Fig. 3.16 Oxidation by TMAO of cytochromes in the membrane fraction of TMAO-grown S. putrefaciens



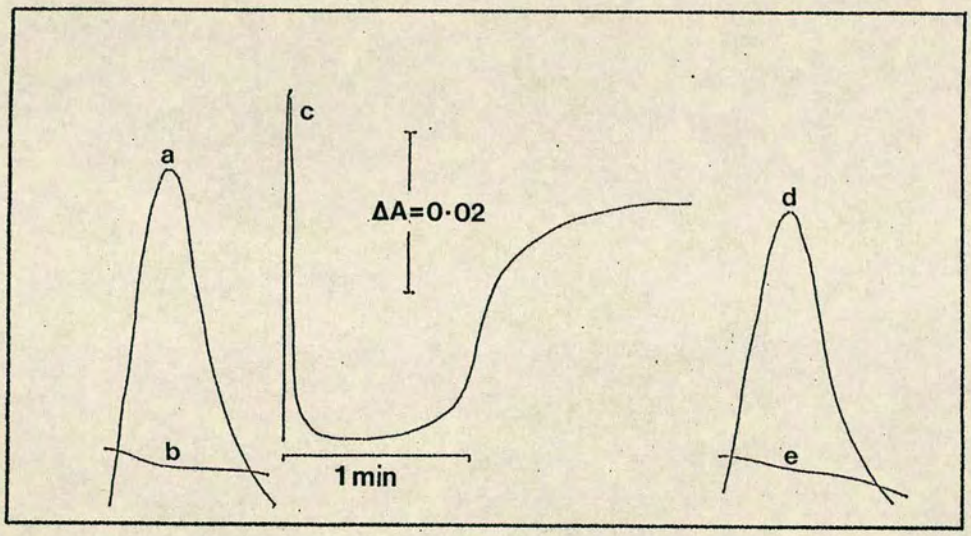
a: baseline. b: dithionite reduced minus oxidised. c: dithionite reduced minus dithionite titrated. d: 3 min after TMAO pulse. e: 9 min after TMAO pulse.

Fig. 3.17 Spectrum of high-potential membrane-bound cytochromes from TMAO-grown S. putrefaciens not oxidised by TMAO



c: baseline. a: dithionite reduced minus oxidised. b and d: fresh preparation titrated with dithionite to 93% reduction. e: TMAO reoxidation. f: ascorbate reduced minus oxidised.

Fig. 3.18 Re-oxidation by TMAO of periplasmic cytochromes from TMAO-grown S. putrefaciens: time scan



a: solid dithionite reduced minus oxidised. b: solid dithionite reduced minus dithionite (titrated) reduced. c: 421 nm scan after TMAO pulse. d: solid dithionite reduced minus TMAO oxidised.

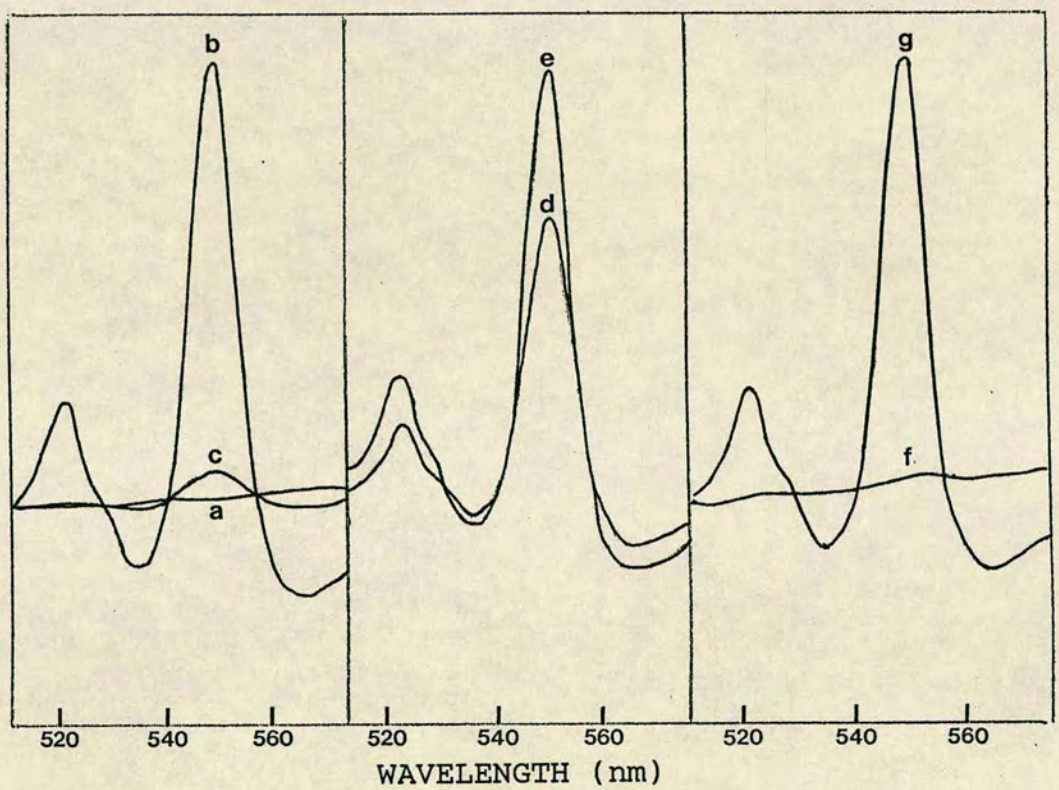
3.1.5 Inhibition of Substrate Oxidation by PCMBS

In the presence of PCMBS (0.57 mM) the rate of TMAO oxidation of the reduced cytochromes was considerably decreased, from the order of seconds in the control to about 25 min in the PCMBS-treated sample (Fig. 3.19). It was concluded from this that an iron-sulphur protein, possibly the TMAO reductase, was present at a site on the oxidising side of the cytochrome.

3.2 SDS-PAGE Profiles With Haem-specific Staining

Whole cells grown aerobically, under oxygen-limiting conditions, and induced with TMAO, fumarate and nitrate, were analysed by SDS-PAGE (7.5-15% gradient gels) with haem staining, as well as subcellular fractions derived from them. The method was used in a qualitative manner in order to establish by molecular weight which cytochromes might be induced under various growth conditions, and to determine the cellular location of the cytochromes. The results are presented in Plates 3.1 to 3.5, and show that there is little qualitative difference between treatments except for aerobic cells, which contained fewer cytochromes. The cytochrome content of the cytoplasms relative to protein content appeared to be appreciably lower than in the periplasmic fractions, when haem and protein

Fig. 3.19 Effect of PCMBS on the reoxidation by TMAO of periplasmic cytochromes from TMAO-grown S. putrefaciens



a: baseline. b: dithionite reduced minus oxidised. c: reduced minus dithionite titrated. d: TMAO reoxidised (with PCMBS, 0·57 mM) after 3 min. e: after 21 min. f: as c. g: TMAO reoxidation control (no PCMBS). Full reoxidation within 3 min.

Plates 3.1-3.5 Peroxidase bands of whole cells from S. putrefaciens separated by SDS-PAGE

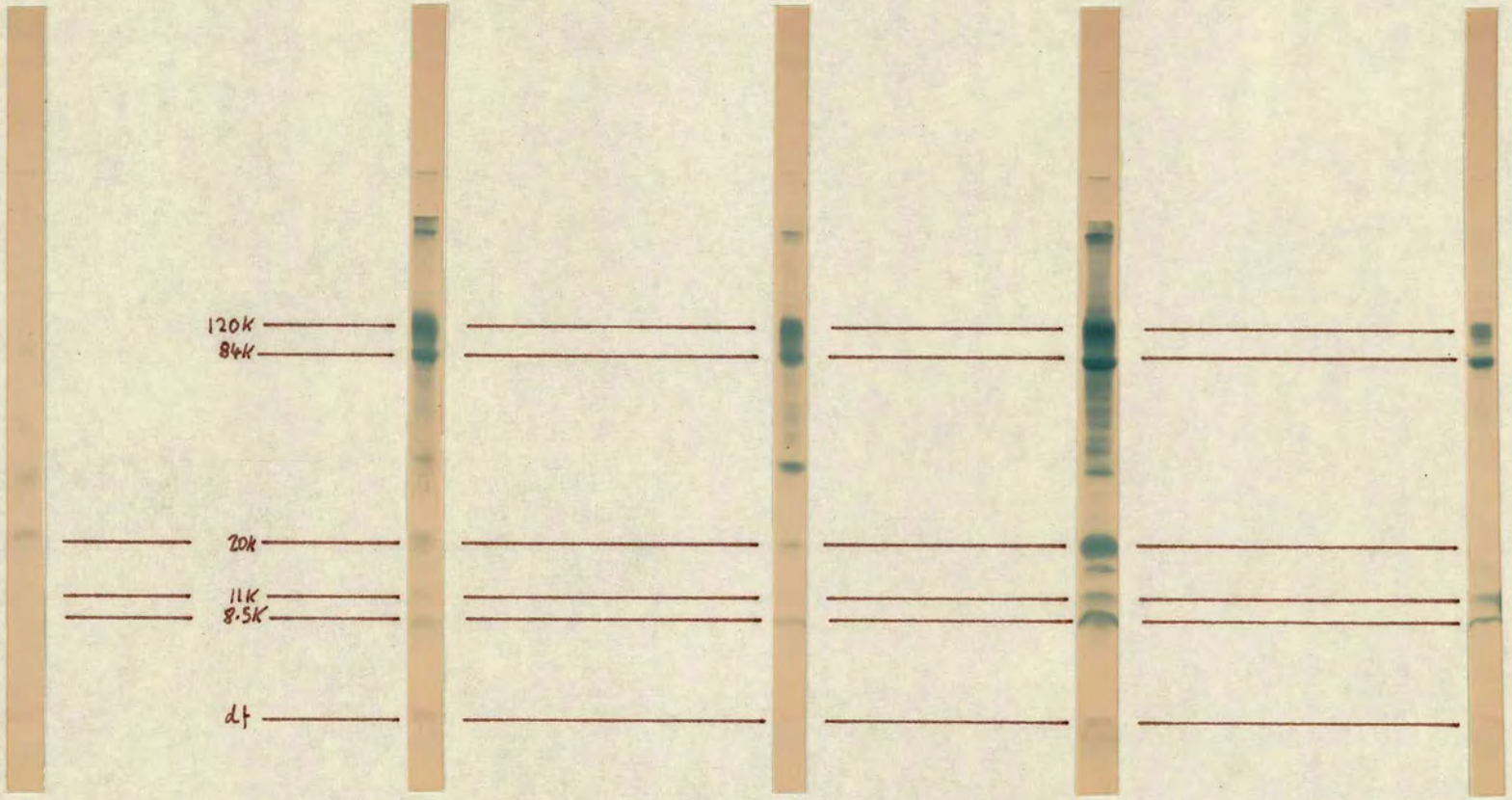


Plate 3.1
Aerobically grown

Plate 3.2
O_2-limited

Plate 3.3
TMAO-grown

Plate 3.4
Fumarate-grown

Plate 3.5
NO_3^--grown

staining were performed on the same gel. The relative molecular weights of the peroxidase stained bands were determined for several batches of cells grown under different conditions.

Aerobic cells contained major cytochromes of 20 000 d and 8 500 d with minor components of 84 000 d, 15 000 d and 11 000 d. The presence of the 84 000 d band correlated with the optical density of the cultures at harvesting: it was rarely detected in aerobic cells grown to A_{660} 0.5, but progressively increased in relative content in cells harvested above A_{660} 0.5.

Oxygen limited and anaerobic induced cells contained the same five cytochromes as aerobically grown cells, at 8 500 d, 11 000 d, 15 000 d, 20 000 d and 84 000 d. The major cytochrome component of these cells was the 84 000 d band. Two additional components were revealed by a broad band of peroxidase activity at 100-120 000 d, and a band which electrophoresed at the dyefront: these were not observed in aerobically-grown cells. The 15 000 d band was a minor component compared with aerobic cells. Minor bands were occasionally observed in anaerobic induced cells at 13 000 d, 32 000 d, 42 000 d, 54 000 d and 200 000 d. Since these bands were not always present they were ignored throughout the rest of this study. The main bands of peroxidase activity from the particulate and soluble fractions of cell batches used in this study

are presented in Fig.3.20 and are summarised in Table 3.4.

3.3 Ion Exchange Profiles of Soluble Fractions

Ion exchange chromatography of soluble cell fractions was used as a method of comparing the cytochrome complement of cells grown anaerobically with different electron acceptors, and under conditions of oxygen limitation. The aims were to compare the elution profiles of the cytochromes present, and to partially characterise the cytochromes resolved by this method on the basis of ion exchange and spectral properties, redox potential (low or high) and molecular weight.

Results are given for periplasm obtained from O_2-limited and TMAO, fumarate and NO_3^--induced cells in Figs. 3.21 to 3.24 respectively. Fig. 3.25 shows the elution profile for a batch of cytoplasms pooled from cells grown under different respiratory conditions.

The profile obtained from the periplasms showed that with each terminal electron acceptor, two major and five minor cytochrome peaks were detected. The two major peaks were designated Peak 4 and Peak 8 and eluted at about 100 and 300 mM NaCl, respectively. The minor peaks were designated Peaks 3 (which eluted in the ferrous state), 3(a), 5, 6 and 7 in order of increasing salt concentration required for elution.

Fig. 3.20 Characterisation of the cytochromes of S. putrefaciens by molecular weight

Several samples from different batches of cells were taken for each growth condition. The cytochromes present in a single sample are represented by a vertical column of dots. Large dots represent major cytochromes; small dots represent minor cytochromes. Major and minor cytochromes were distinguished visually.

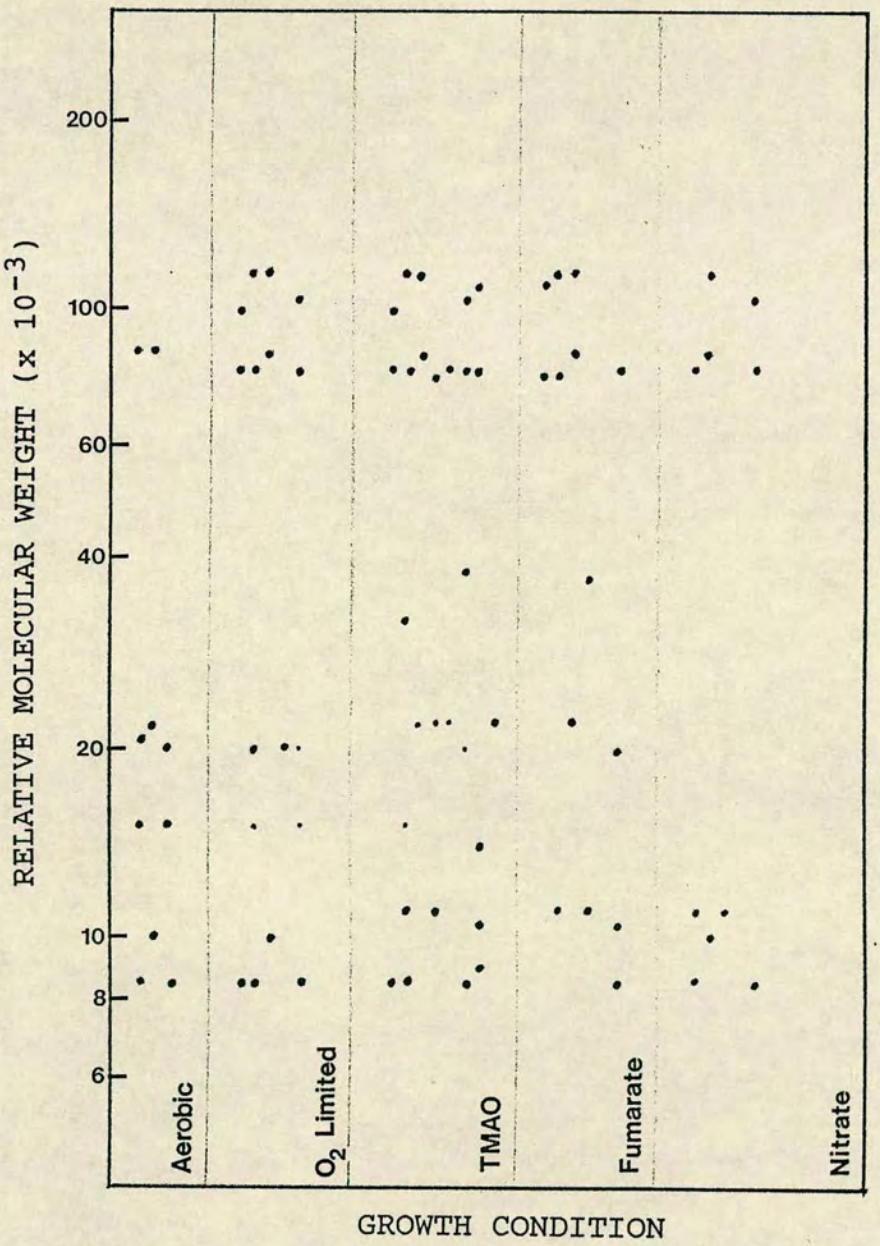


Table 3.4. Main bands of peroxidase activity found in cell fractions of S. putrefaciens grown under different conditions

Growth condition		Mr x 10^{-3} dyefront	8.5	11	15	20	84	100	120
Aerobic	M		+++	++	+	+++			
	P		++	++			+		
	C		+	+					
O_2-limited	M		++	++	+	++	+		
	P		++	++			+++	++	++
	C		+	+			+		
TMAO	M		++	++	+	++	+	++	++
	P		++	++			+++		
	C		+	+			+		
Fumarate	M		++	++	+	++	+	++	++
	P		++	++			+++		
	C		+	+			+		
Nitrate	M		++	++	+	++	+		
	P		++	++			+++	++	++
	C		+	+			+		

M = membrane, P = periplasm, C= cytoplasm

Fig. 3.21 Ion exchange profile of the periplasmic fraction of oxygen-limited S. putrefaciens

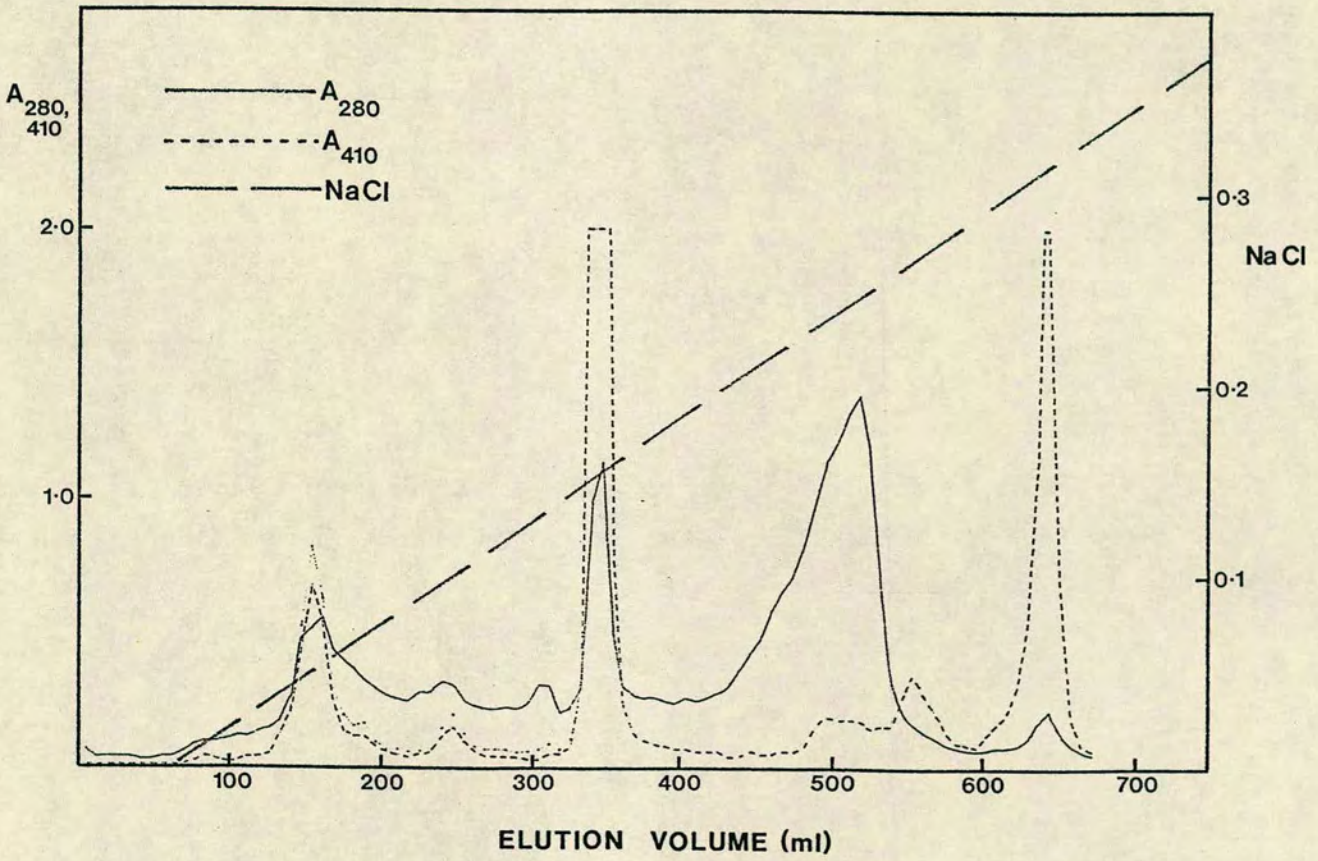


Fig. 3.22 Ion exchange profile of the periplasmic fraction from TMAO-grown S. putrefaciens

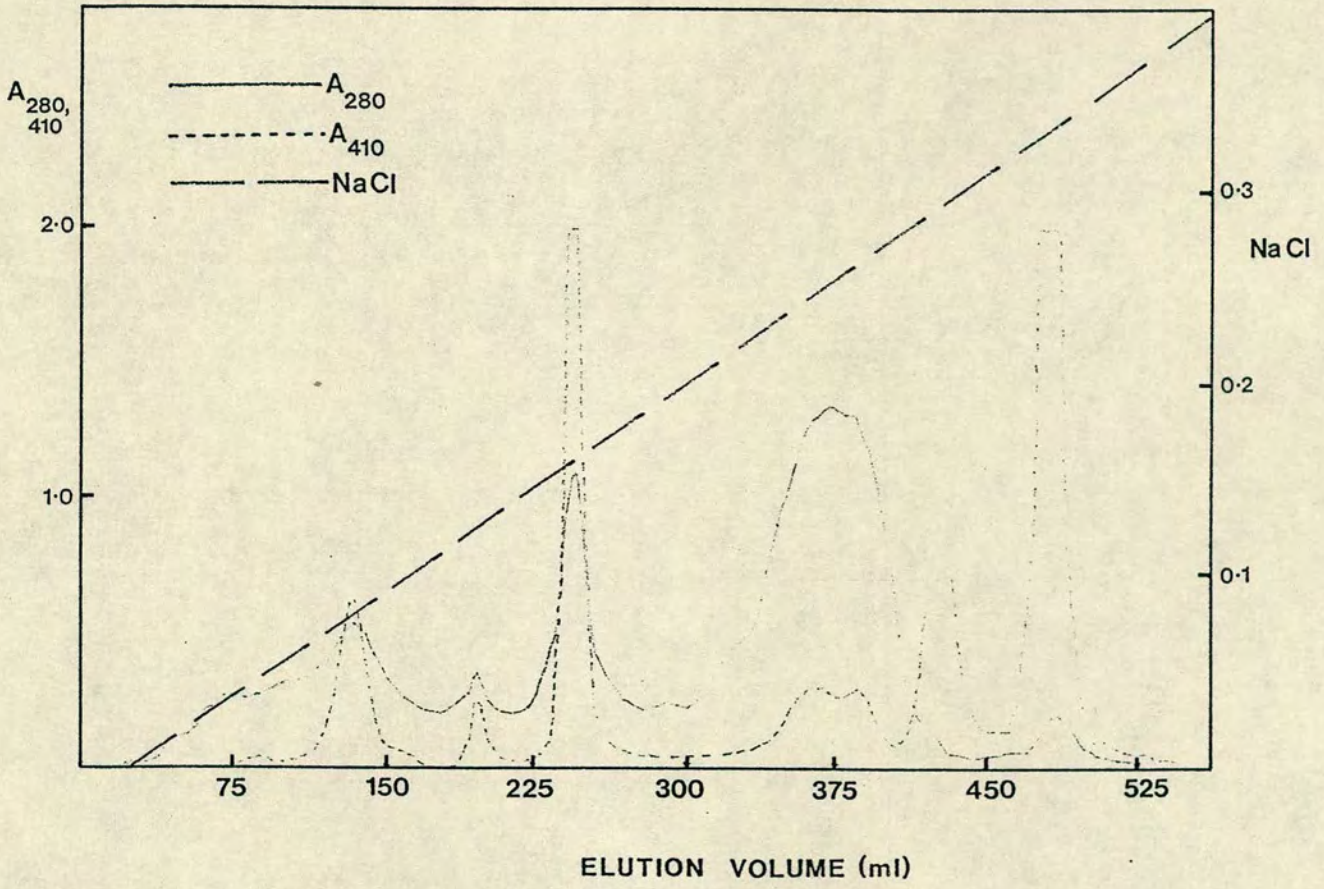


Fig. 3.23 Ion exchange profile of the periplasmic fraction from fumarate-grown S. putrefaciens

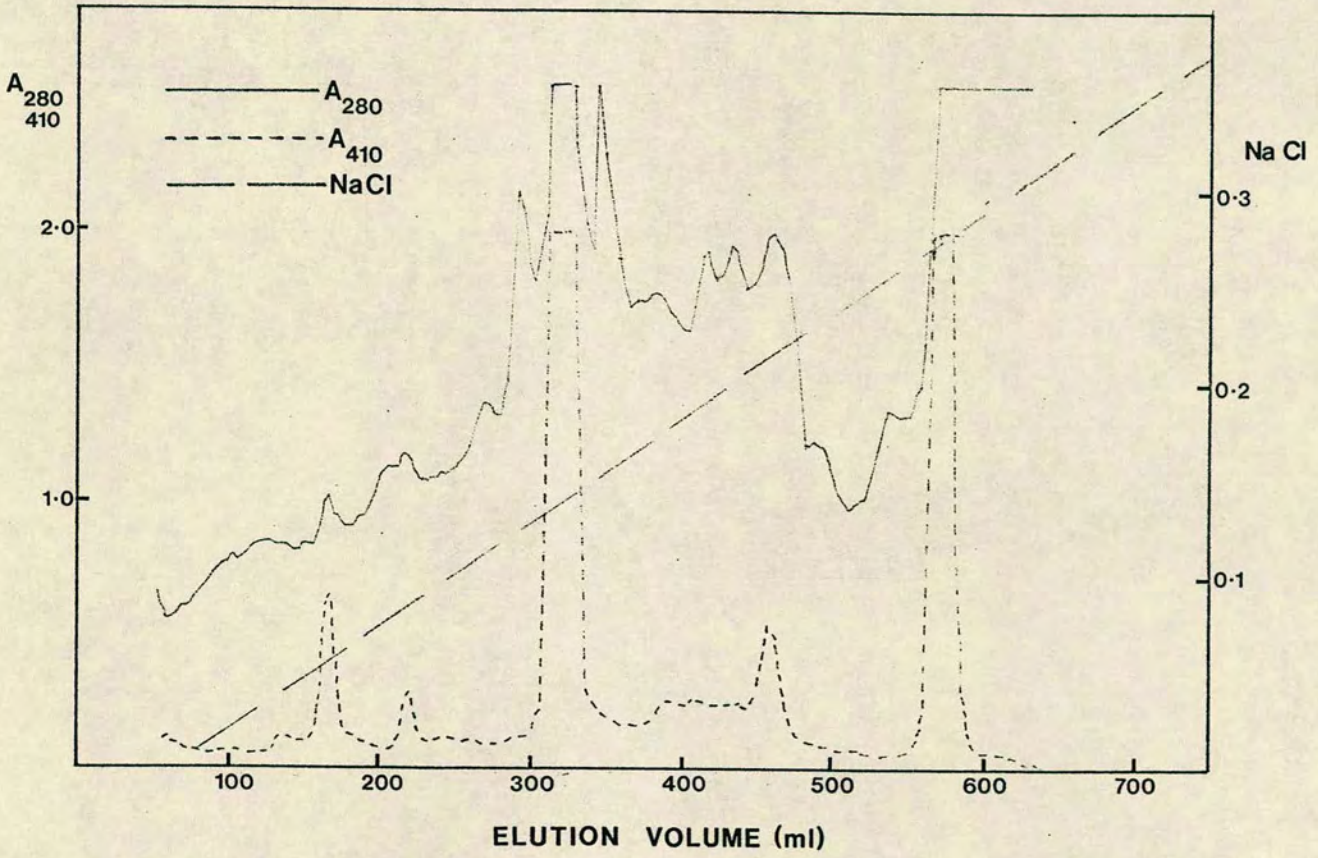


Fig. 3.24 Ion exchange profile of the periplasmic fraction from nitrate-grown S. putrefaciens

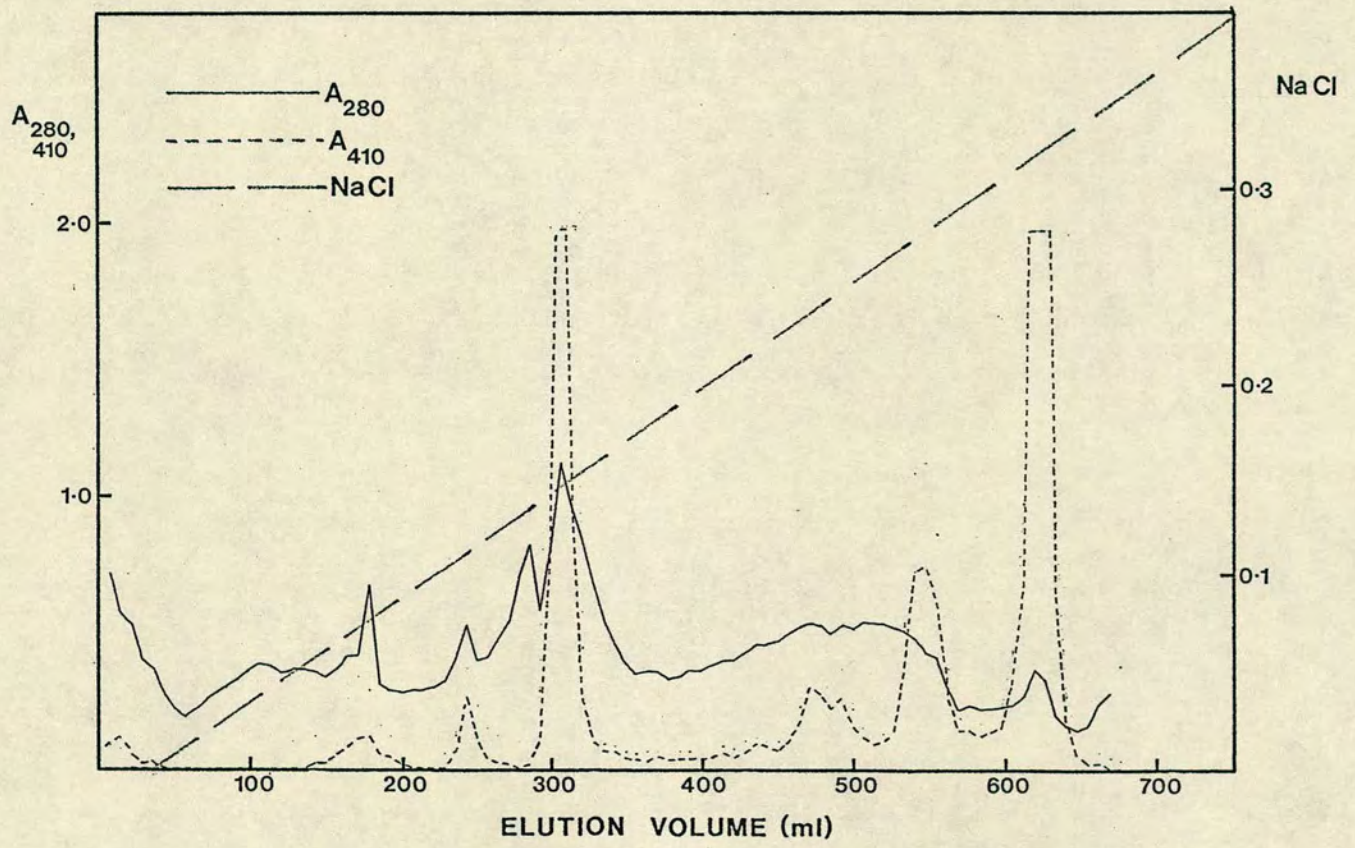
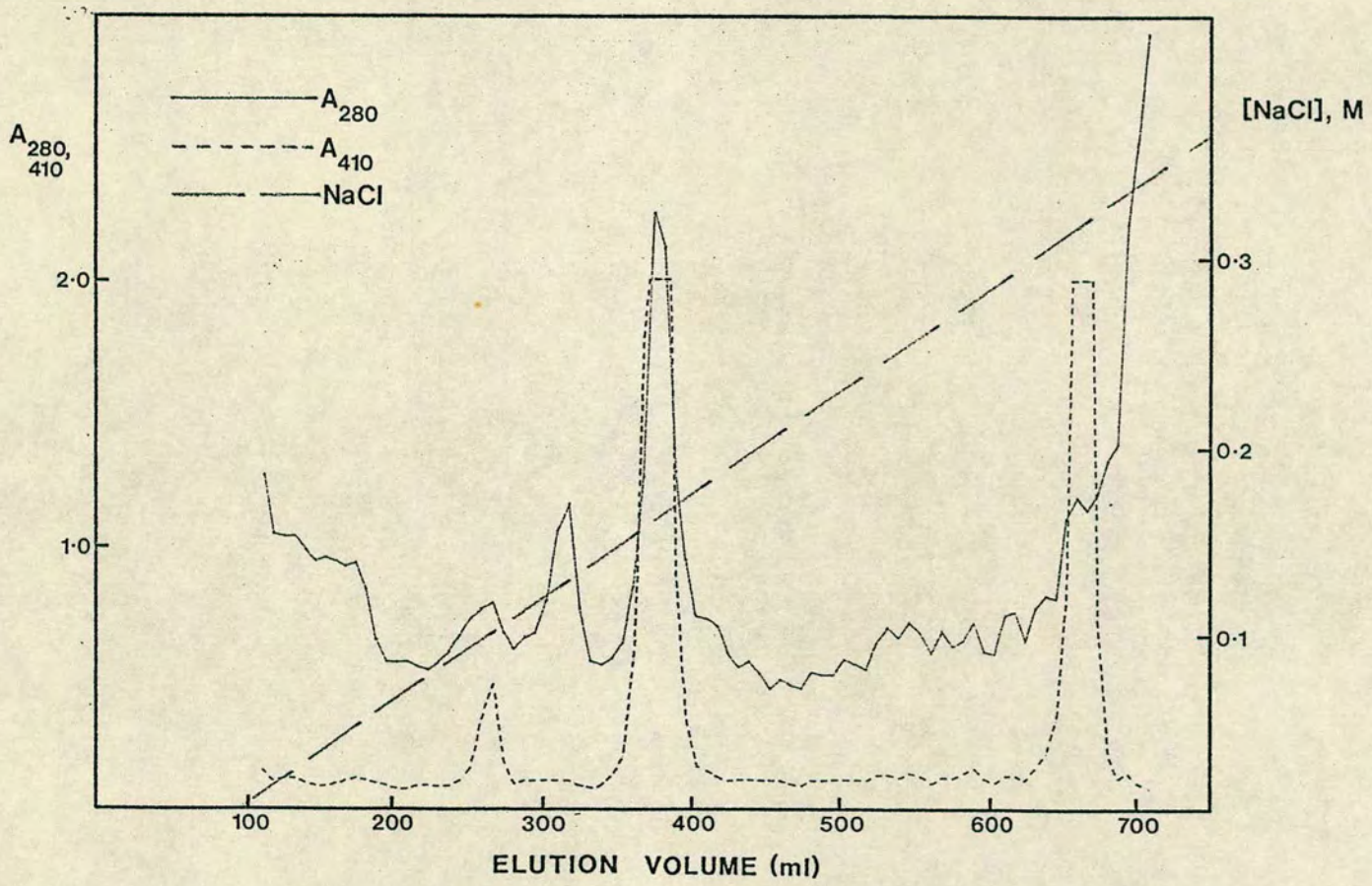


Fig. 3.25 Ion exchange profile of the cytoplasmic fraction from anaerobically-grown S. putrefaciens



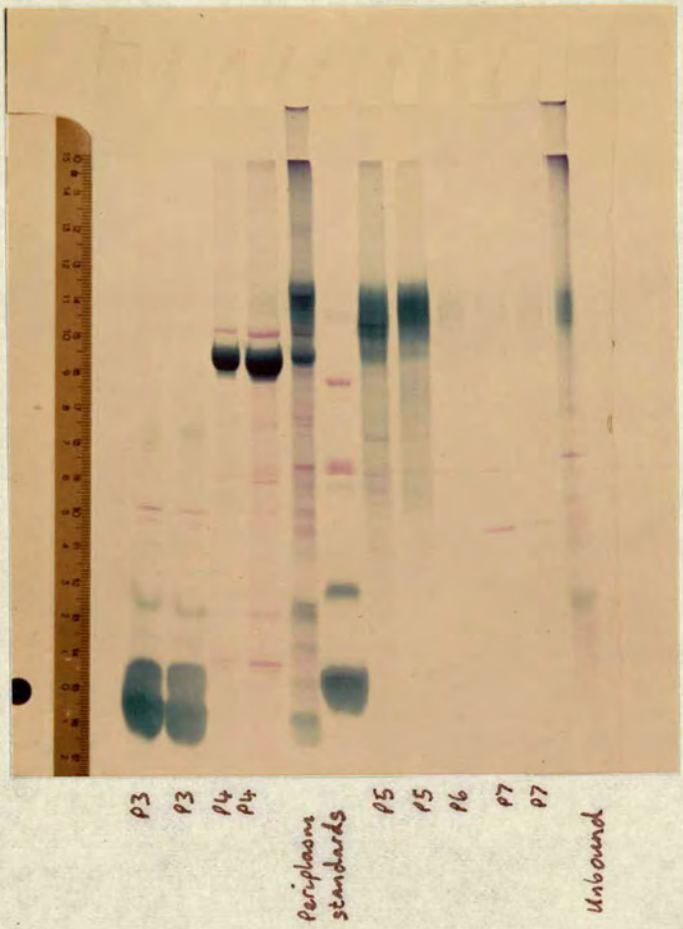
The mixed cytoplasm profile contained only Peaks 3, 4 and 8 possibly indicating that these cytochromes were loosely bound to the outside of the inner cell membrane while Peaks 3(a), 5, 6 and 7 were truly periplasmic. Evidence that peaks detected in cells grown under one respiratory condition were the same as those from cells grown with a different electron acceptor was obtained by electrophoresing samples from the peaks in the presence of SDS and staining for haem. In every case, the molecular weights of the cytochrome peaks from each respiratory condition were the same (see Table 3.5), and corresponded well with the bands of peroxidase activity found in the soluble fractions before chromatography (Plate 3.6).

A proportion of the cytochrome content from each soluble fraction did not bind to the ion exchange column. SDS-PAGE analysis resolved two bands of peroxidase activity in this unbound fraction at 20 000 d and at ≈100 000 d, together with a large number of contaminating nonhaem proteins (Plate 3.6). Attempts to separate and further purify these two cytochromes by hydroxyapatite column chromatography were frustrated by rapid formation of a precipitate in the fraction upon standing. This problem was avoided by batch adsorption of the fractions to hydroxyapatite gel prepared as for column chromatography. The 20 000 d cytochrome was bound to the gel and eluted in the ferrous state with 2.0 M phosphate pH 8.4 after

Table 3.5. Molecular weights of cytochromes resolved by ion exchange chromatography.

Ion exchange peak	Molecular weight of haem-staining band x 10^{-3}
3	8.5, 11
3(a)	11, 15
4	84
5	100
6	100
7	120
8	dyefront

Plate 3.6 Analysis of haem-containing ion exchange peaks by SDS-PAGE and haem staining



removal of unbound protein including a 100 000 d cytochrome, by washing with T- buffer (see Section 4.3.3).

The variations in cytochrome content within and between treatments was not very great and there was no strong evidence to support the contention that a particular cytochrome was induced in the presence of only one terminal electron acceptor. In fact, with the exception of fumarate-grown cells which had a greater overall cytochrome c concentration, the relative cytochrome content of cells grown under different conditions would suggest that either Peaks 3, 3(a), 4, 5, 6, 7 and 8 (as well as the unbound 20 000 d and 100 000 d bands) were all involved in electron transport on any particular terminal substrate, or that several different electron transport systems were induced and at least potentially capable of operating in parallel.

Given that TMAO was incapable of oxidising high-potential periplasmic and particulate cytochromes (Section 3.1.2) and that Peak 3 and the 20 000 d unbound cytochrome were ascorbate reducible and therefore excluded from fumarate respiration (Em_7 fumarate/succinate = +33 mV; ascorbate/dehydro-ascorbate = +80 mV) it would appear that Peak 3 and the 20 000 d band are exclusively involved in aerobic respiration.

3.4 Redox Titrations

Mixtures of cytochromes with similar spectral properties may be resolved by stepwise reduction of the mixture if the redox potentials are recorded with the spectra at each step. Correlation of the midpoint redox potential of a purified cytochrome with the redox potential obtained from such a mixture is generally agreed upon as being a reliable indication that the cytochrome has not been altered by the purification procedure. The midpoint potential of cytochromes has been used extensively as a criterion for tentative siting of the species within electron transport sequences. All of these features were considered in this study.

For initial exploratory work starting with preparations containing an unknown number of cytochromes described as high-potential or low-potential on the basis of their reaction with ascorbate or dithionite, experimental conditions were Systematically examined and altered to give optimal conditions for the system being studied. Particular attention was given to the effects of the following experimental parameters:

i) Redox mediators. These were chosen to cover the predicted Em span of the cytochromes being studied. For exploratory work a large number of mediators covering a wide span were employed.

ii) Buffer type and concentration. A strong buffer active at the operative pH was chosen and used at a concentration high enough so that any pH changes which might possibly occur, e.g. acidic breakdown products of dithionite, were resisted.

iii) Choice of reductant and oxidant. Dithionite and ferricyanide were used respectively.

Initial experiments exposed two major problems. Firstly, when periplasm was titrated, the experiments were never completed due to a drastic alteration of baseline which developed after a period of time during the titration. This baseline shift was found to be caused by a white precipitate which appeared first in the oxidised reference cell and was dependent upon the concentration of the added phosphate buffer. Decreasing the concentration from 0.5 M to 50 mM had no effect. At 30 mM the time taken for precipitate formation was increased, and with no added phosphate no precipitation occurred. The problem was solved by decreasing the concentration of Mg^{2+} used for salting out EDTA during spheroplasting from 50 mM to 5 mM, and adding 5 mM EDTA after removal of spheroplasts.

Since previous results (Sections 3.2 and 3.3) showed that no great quantitative or qualitative difference existed in the cytochromes present in cells of S. putrefaciens grown under a variety of conditions, the soluble fractions from TMAO-induced cells were selected for redox potentiometric analysis. Samples of

cytochrome and periplasm were taken to 100 mM phosphate pH 7.0 using 2.0 M phosphate pH 7.0. The following redox mediators were added to a final concentration of 10 uM: A2S, A26D, HNQ, BV and DQ. Dithionite was dissolved in 500 mM phosphate pH 7.0 in aliquots of concentration varying from 20 mM to 200 mM, and used such that additions of 2-5 ul to the reaction cuvette were adequate for suitable increments in redox potential. The redox spectrum obtained by titration of the periplasmic fraction showed a single symmetrical α-peak at 552 nm which remained consistent throughout the titration (Fig. 3.26).

The corresponding plots of A_{552} vs Eh and $\log_{10}$ [ox]/[red] vs Eh (Figs. 3.27 and 3.28) indicate the presence of several haems of different potential, about 87% low-potential and 13% high-potential. The high-potential species was estimated from Fig. 3.27 to have a redox potential of above 100 mV. The low-potential haems were mathematically resolved into three species (Fig 3.29) with redox potentials of about -93 mV, -216 mV and -329 mV, with relative contributions to total haem content of 30%, 21% and 36% respectively.

The results obtained by titration of the cytoplasmic fraction were very similar, with a symmetrical α-peak at 552 nm (Fig. 3.30), a single high-potential species at above 200 mV contributing about 10% towards the total absorbance at 552 nm (Fig.

Fig. 3.26 Redox spectrum of the periplasmic fraction from TMAO-grown S. putrefaciens

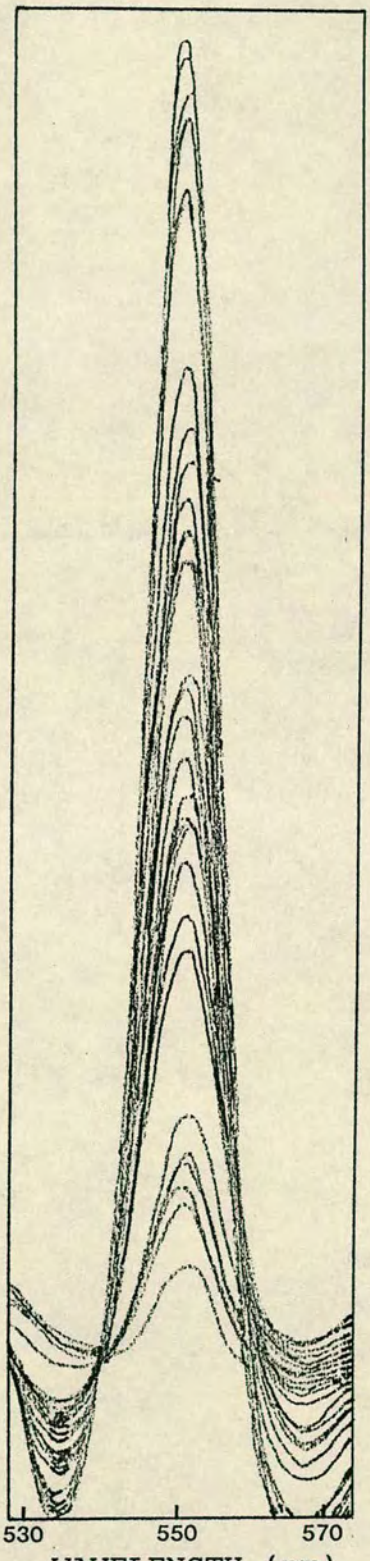


Fig. 3.27 Plot of % reduced vs ambient redox potential

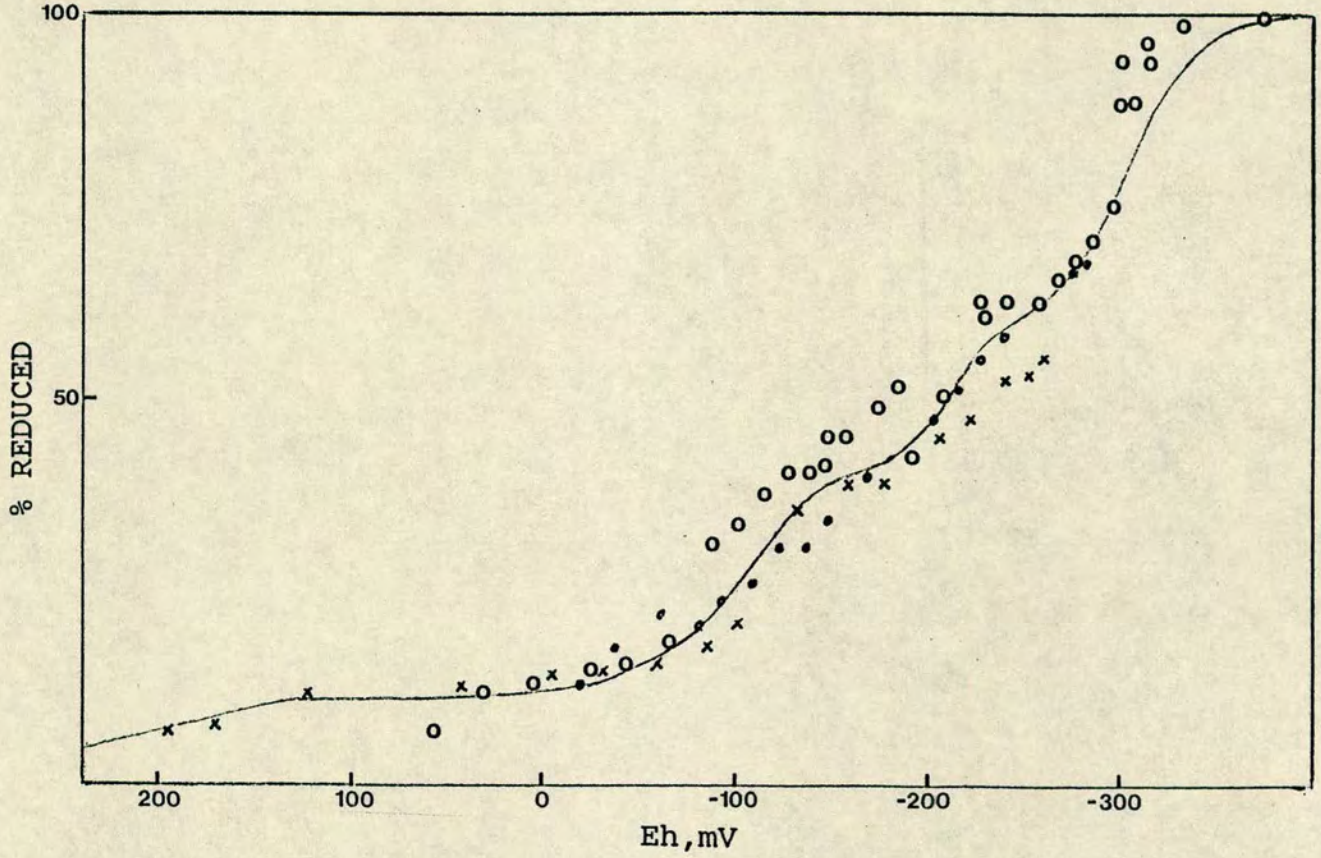


Fig. 3.28 Nernst plot of the redox titration of the periplasmic fraction from TMAO-grown S. putrefaciens

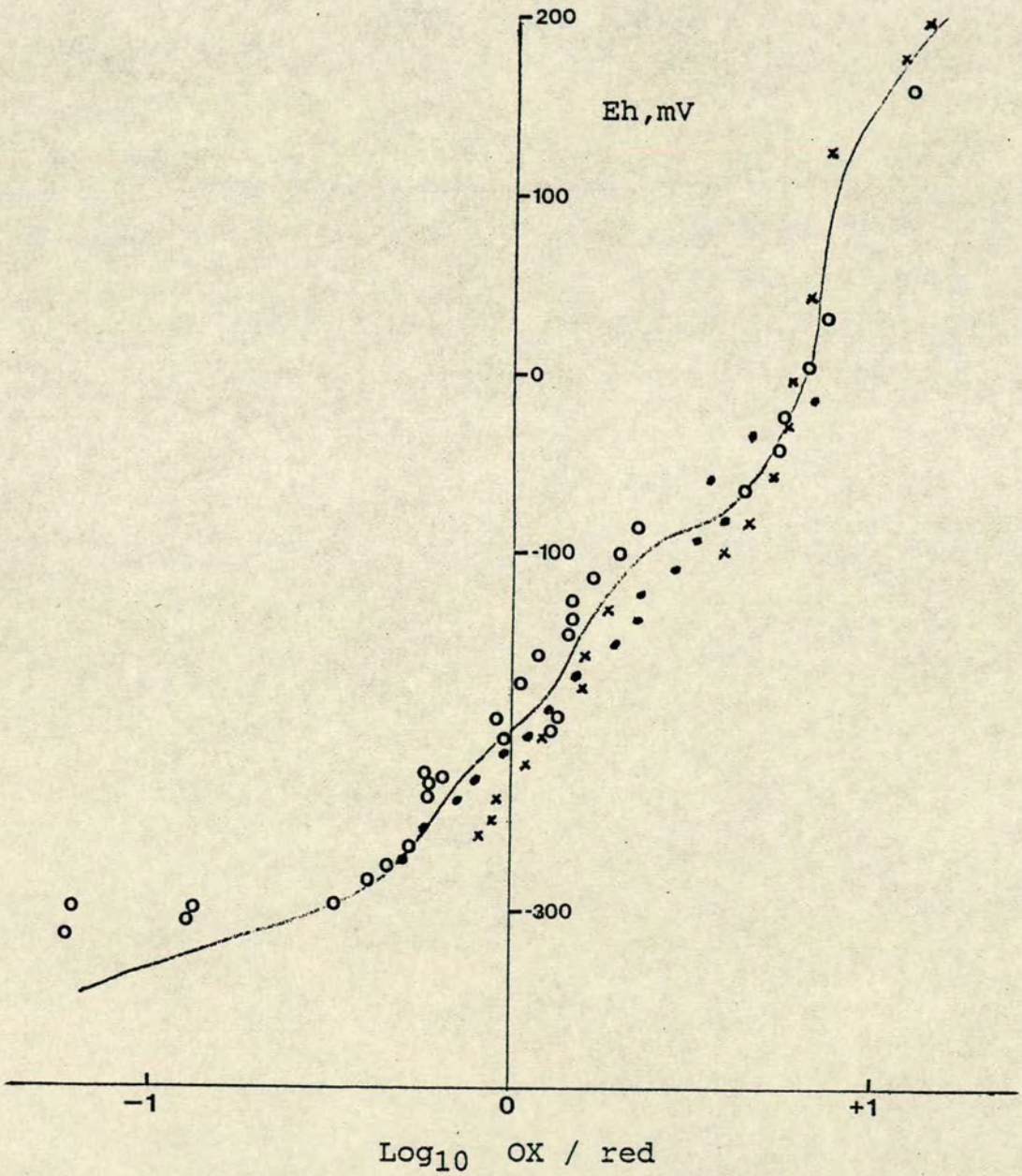


Fig. 3.29 Three-component resolution of the Nernst plot

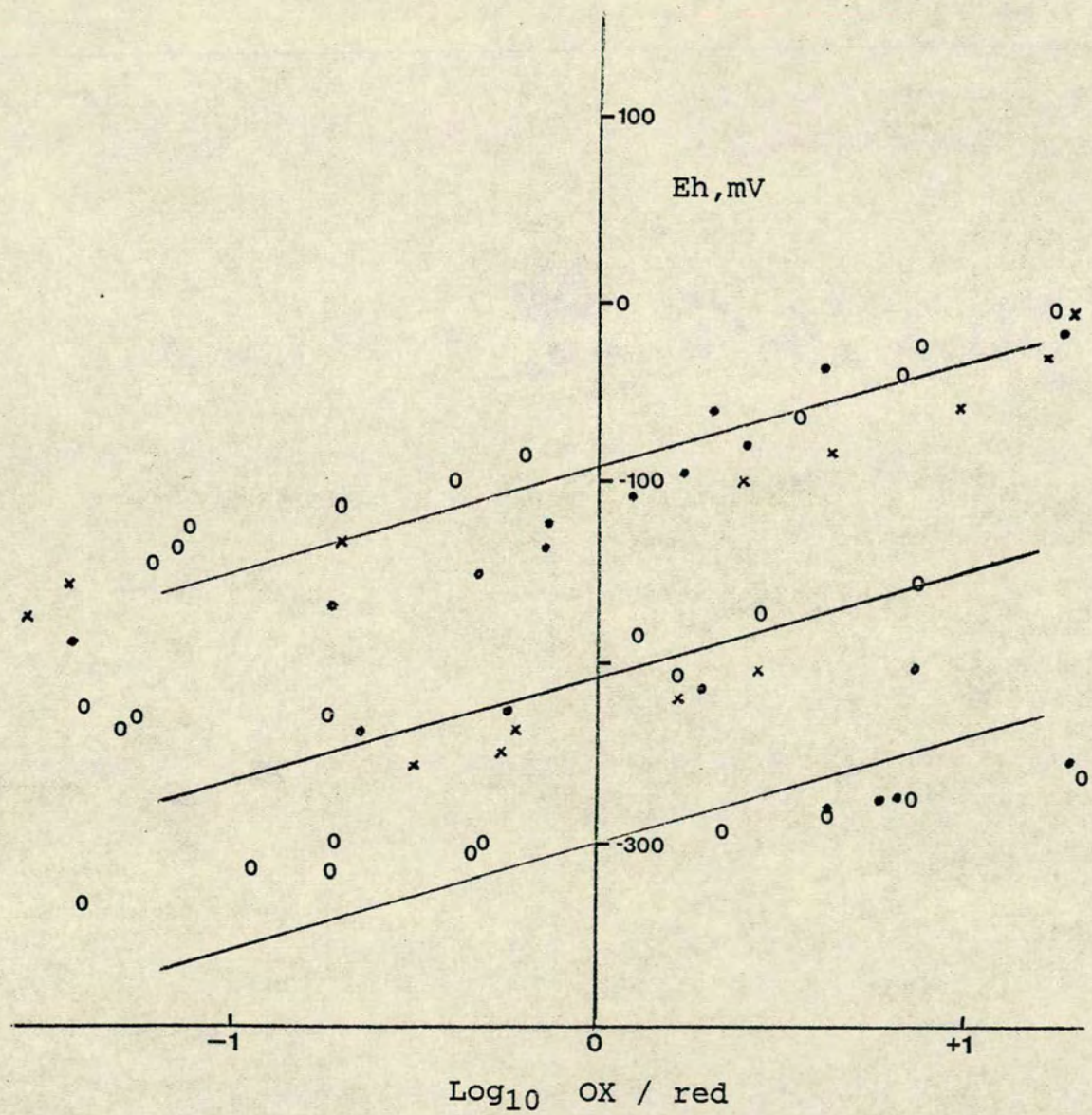
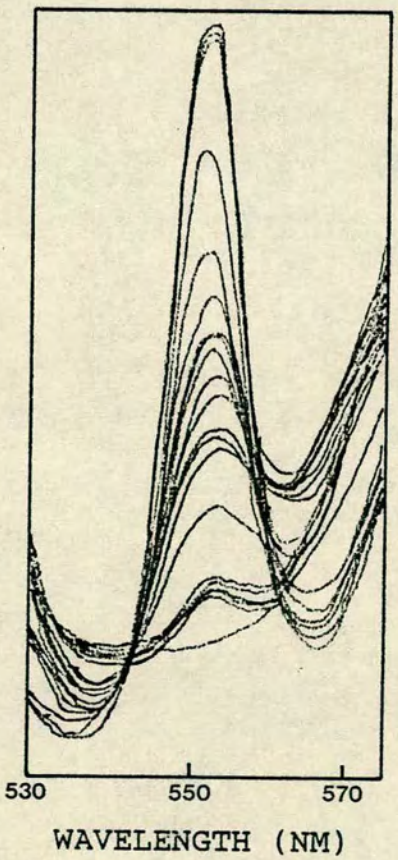


Fig. 3.30 Redox spectrum of the cytoplasmic fraction from TMAO-grown S. putrefaciens



3.31) and three low-potential haems with potentials of -72 mV (22%), -171 mV (22%) and -267 mV (46%) (Figs 3.32 and 3.33).

Some difficulties were encountered during the course of reductive titrations below about -100 mV. Following a pulse of reductant the absorbance increased rapidly to its maximum while the measured ambient redox potential dropped slowly. Over a period of 5-15 min the absorbance at 552 nm slowly decreased to a stable level, followed by or concomitant with changes in Eh, which slowly decreased to a lower level than before. Increasing the concentration of mediators had little effect, so titrations often took 4-5 h to perform with unknown consequences to the physicochemical characteristics of the cytochromes. It would appear likely that a component present in the preparation was reacting with breakdown products of dithionite (ElKurdi et al., 1982).

The results are in general agreement with those in Table 3.1 except that the proportion of high-potential haem in the samples used for redox titrations was about 3-fold higher than that measured for chemical reduction analysis. It was known, however, that significant differences in the relative concentrations of cytochromes can be observed in different batches of cells grown under the same conditions.

Accurate midpoint potentials for the cytochromes in the crude cell fractions could not be obtained

Fig. 3.31 Plot of % reduced vs ambient redox potential

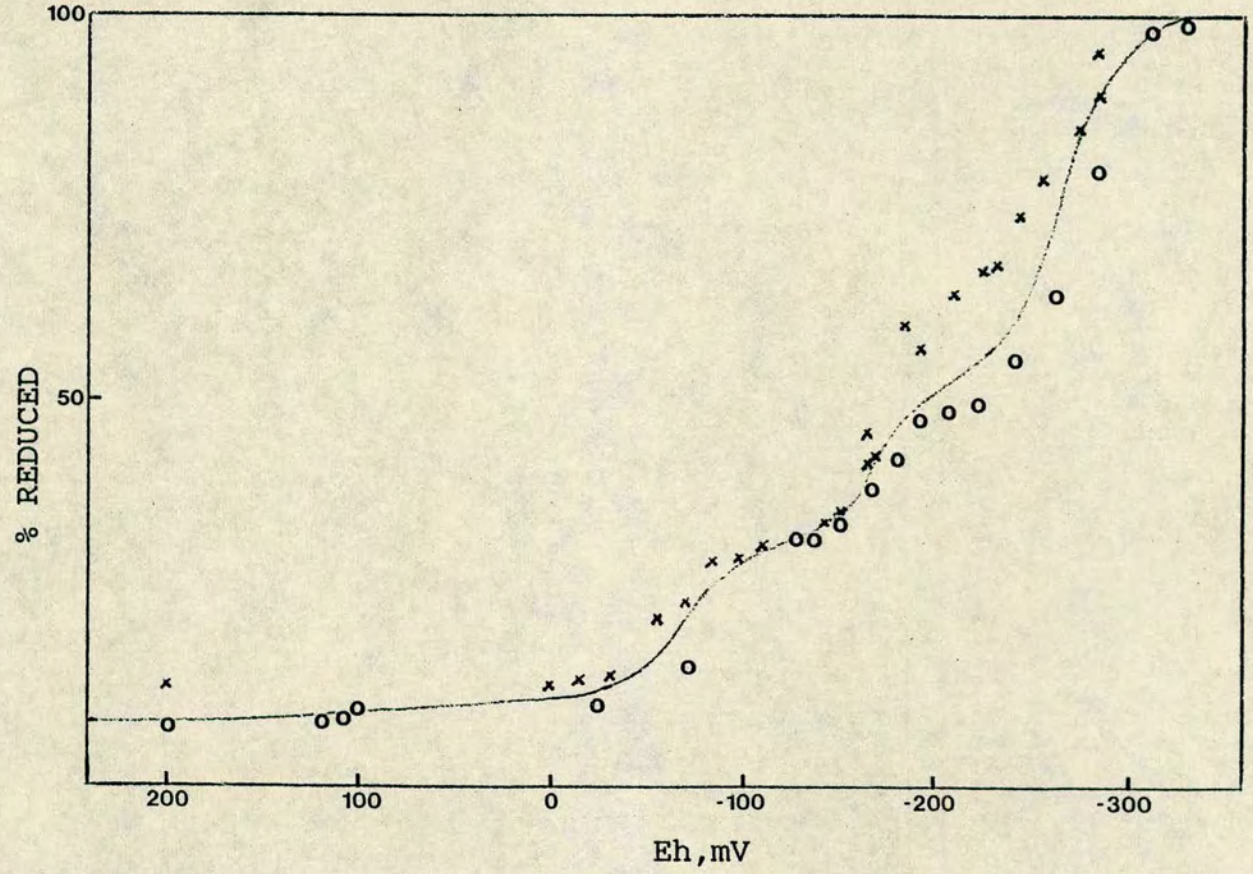


Fig. 3.32 Nernst plot of the redox titration of the cytoplasmic fraction from TMAO-grown S. putrefaciens

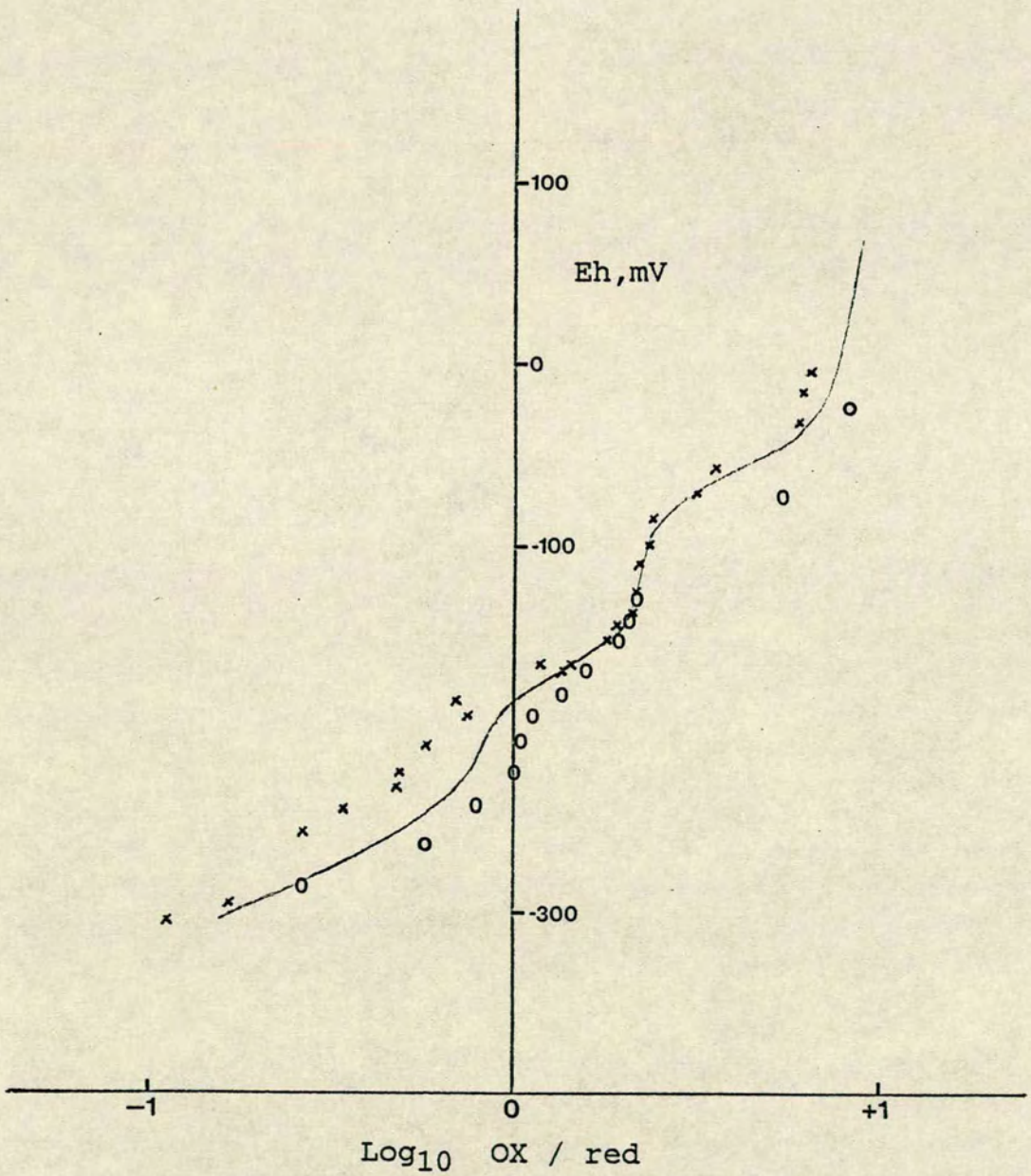
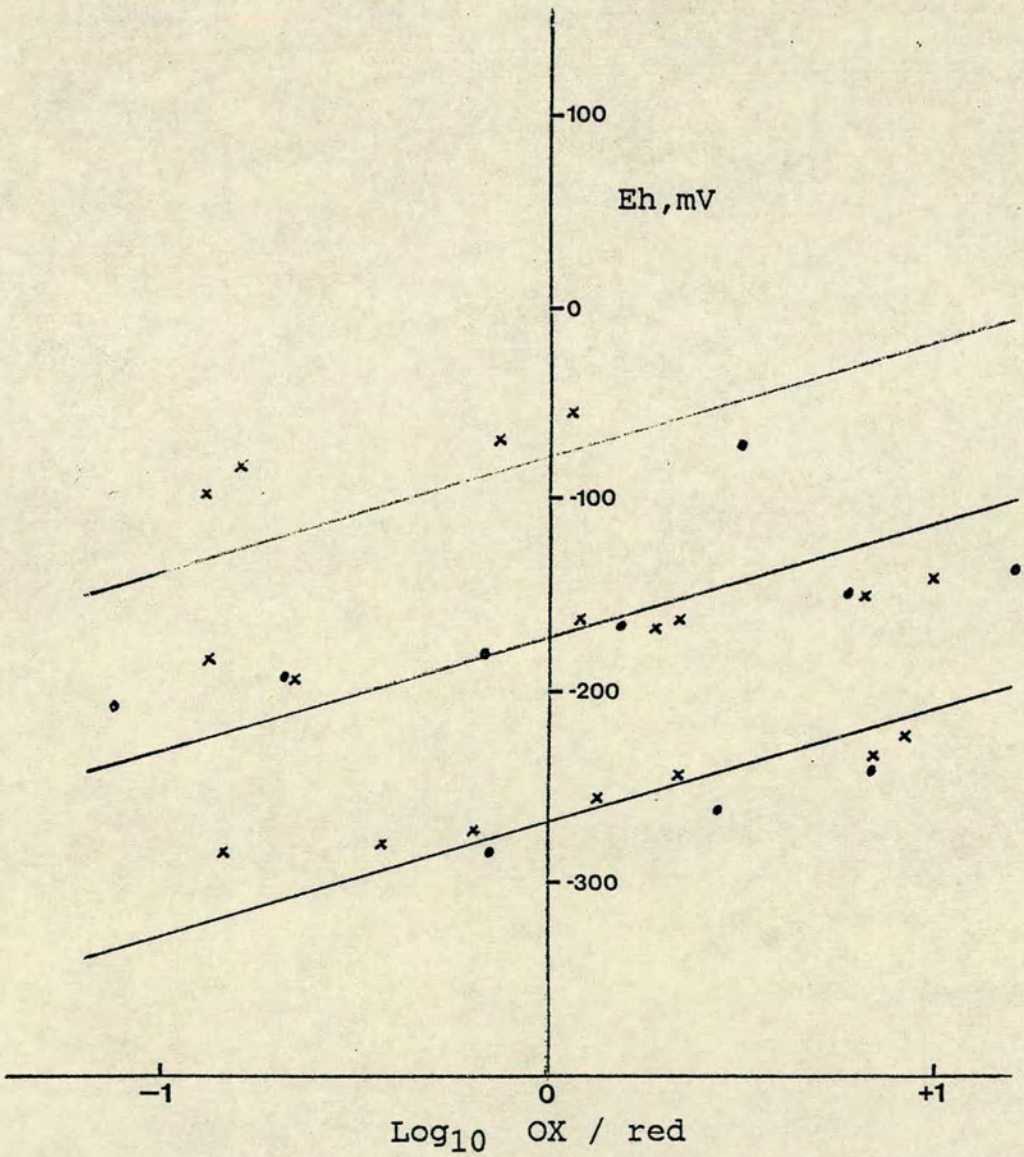


Fig. 3.33 Three- component resolution of the Nernst plot



because the potentials were not widely separated. More accurate estimates would need purified preparations. An additional complication is that the presence of two redox centres does not necessarily indicate the presence of two cytochromes as a single cytochrome may have haem groups with different midpoint potentials. These two possibilities cannot be resolved by redox titrimetry alone.

3.5 Formate Dehydrogenase

3.5.1 Formate Dehydrogenase Assayed With Different Electron Acceptors

Formate was found to be the most effective electron donor to low-potential cytochromes found in oxygen-limited and induced cells of S. putrefaciens. Reduction of the cytochromes by formate was inhibited by PCMBS, HQNO and Antimycin A. In order to establish the sites at which the inhibitors act, the formate dehydrogenase of membranes from induced cells was assayed for activity using several artifial and physiological electron acceptors, in the absence and presence of these inhibitors. The following exogenous electron acceptors were used:

a) phenazine methosulphate (PMS), a flavin analogue which might be expected to oxidise a flavoenzyme.

b) methylene blue (MB) which in the mitochondrial respiratory chain and in some bacteria, e.g.

Cornyebacterium diphtheriae (Scholes & King, 1965) receives electrons at the level of cytochrome *b* and has a redox potential (+29 mV) compatible with the low-potential components of respiratory chains, e.g. it cannot be reduced by mitochondrial cytochrome *c*.

c) ferricyanide (FIC) accepts electrons from cytochrome *c* in the mitochondrial respiratory chain and can be used to assay electron flow through the bc_1 complex providing catalytic quantities of cytochrome *c* are present (Beattie & Villalobo, 1982). In microbial systems FIC appears to favour FeS clusters and can be reduced by the formate dehydrogenase and the fumarate reductase enzymes of *Vibrio succinogenes* (Kroger, 1980).

d) Mammalian cytochrome *c* was chosen as a control electron acceptor; it was assumed that a quinone-cytochrome *c* oxidoreductase would be required for reduction in constrast with the potentially unpredictable sites of the other electron acceptors.

The enzyme source was spheroplasts and membranes collected from induced spheroplasted cells: after optimising experimental conditions the enzyme concentration was held constant. The specific activities of each electron acceptor with formate dehydrogenase are given in Table 3.6. As all the

Table 3.6. Inhibition of the formate dehydrogenase of S. putrefaciens assayed with different electron acceptors.

Electron acceptor	Specific activity umol min^{-1} mg $protein^{-1}$		% Activity with inhibitor (spheroplasts = 100%)			
	Membranes	Spheroplasts	PCMBS	Anti.A	HQNO	Ethanol (control)
Phenazine methosulphate	4.17	395	80	47	93	88
$K_3Fe(CN)_6$	25.6	127	52	109	104	nd
Methylene blue	73.7	614	1	39	1	93
Cytochrome c	13.5	500	55	45	27	nd
Flavocytochrome c	1	107	nd	nd	nd	nd

nd = not determined

electron acceptors were active with the formate dehydrogenase, few conclusions may be drawn. (a) Reduction of methylene blue may indicate the presence of a low-potential cytochrome b, in contrast with previous results (Sections 3.1 and 3.2). (b) FIC may have been accepting electrons either from an FeS group, possibly within the FDH enzyme itself, or it may have been reacting with a cytochrome c which was reduced by the FDH. (c) Mammalian cytochrome c may have been reacting with a residual aerobic bc_1-type complex, competing with the endogenous cytochrome. (d) Mammalian cytochrome c reacted far more rapidly with the outside of the membrane (i.e. spheroplasts) than the inside.

3.5.2 Effect of Inhibitors on FDH Activity

More information was obtained by assaying FDH with different electron acceptors in the presence of the inhibitors HQNO, Antimycin A and PCMBS (Table 3.6). The results, presented diagramatically in Fig. 3.34, show the following:

a) Methylene blue and cytochrome c activities were both inhibited by all three inhibitors, indicating that both electron acceptors receive electrons at a site downstream from the sites of inhibition.

b) Ferricyanide activity was inhibited by PCMBS but not by HQNO or Antimycin A. This might support

the contention above that FIC accepts electrons from an FeS centre in the enzyme. The titre for 100% inhibition was determined using concentrations of PCMBS from 10 uM to 2.86 mM and was found to be 100 umol.g protein^{-1}, about one-sixth that of the fumarate reductase of Wolinella succinogenes (Kroger & Innerhofer, 1976).

c) PMS activity was inhibited by Antimycin A but not by HQNO, suggesting that the sites of inhibition of Antimycin A and HQNO are different.

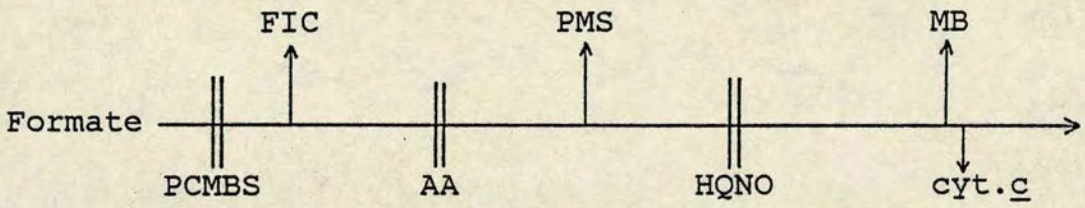


Fig. 3.34. Inhibition pattern of S. putrefaciens formate dehydrogenase

3.6 Discussion

3.6.1 Characterisation of Cytochromes in S. putrefaciens

The purpose of the work described in this chapter was to obtain a view of the cytochromes present in

cells grown on TMAO, and to relate this to the cytochromes found in aerobically-grown cells, and cells grown on different terminal electron acceptors. By a partial characterisation of the cytochromes, enabling them to be identified in different cell extracts, it was thought that those cytochromes which were unique to TMAO respiration might be identified for further study.

The results raised several major points.

a) The identified cytochromes characteristic of aerobically-grown cells persisted into microaerobic growth.

b) No qualitative difference was observed in the cytochrome content of cells grown under conditions of oxygen limitation, or anaerobically on TMAO, fumarate or nitrate.

c) The high-potential cytochromes characteristic of aerobic growth which were found in anaerobic-induced cells were not oxidisible by TMAO, and on the basis of redox potential could not be oxidised by fumarate.

d) The cytochromes fell into two groups on this basis: high-potential aerobic cytochromes, and low-potential anaerobic cytochromes.

e) No low-potential cytochrome b was observed spectrally, although the presence of low-potential cytochrome b was indicated by inhibition studies with formate dehydrogenase. This would suggest either that formate dehydrogenase in S.

putrefaciens does not have a cytochrome b subunit, or that the concentration was too low for detection by the methods described here.

A number of cytochromes were partially characterised (Table 3.7). Two cytochromes, peaks 3 and 3a, were easily identified in cell extracts by their molecular weights of 8 500 d and 11 000 d respectively. Peak 3 was described as high-potential since it eluted as the ferrocytochrome and was ascorbate-reducible. Peak 4 was identified by a molecular weight on SDS/PAGE gels of 84 000 d. Peaks 5, 6 and 7 were difficult to distinguish, since they were all low-potential and spectrally similar, and on SDS-PAGE gels they each gave a smear of peroxidase activity at about 100-120 000 d. Peak 8, a low-potential cytochrome, gave a band of peroxidase activity at the dyefront suggesting loss of haem.

A second high-potential cytochrome with a relative molecular weight of 20 000 d was found in the unbound fraction from ion exchange chromatography, together with at least one cytochrome with a molecular weight of 100-120 000 d. Since this cytochrome was not always solubilised, it is suggested that peak 3 was the major soluble high-potential c-type cytochrome, and that the 20 000 d band was the major high-potential membrane-bound cytochrome. No indication was found of a cytochrome with a split α-peak in soluble cell extracts, and no cytochrome was found with an α-peak of

Table 3.7. Comparison of cytochromes c analysed by ion exchange chromatography and SDS-PAGE.

Molecular weight x 10^3 of observed bands	Ion exchange fraction	General location	Comments
8.5	Peak 3	Soluble	High-potential
11	Peak 3, Peak 3(a)	Soluble	
15	Peak 3(a)	Soluble	Low-potential
20	Unbound	Particulate	High potential, binds tightly to HIC, loosely to HA
84	Peak 4	Soluble	Flavocytochrome c
100	Peak 6/unbound	Periplasmic	2 different types
120	Peak 7/unbound	Periplasmic	Low-potential
Dyefront/32 (HPLC)	Peak 8	Soluble	Low-potential multihaem (c_3)

HIC: hydrophobic interaction chromatography
HA: hydroxyapatite chromatography

548 nm. More information may have been obtained by recording spectra at 77°K. The component(s) which titrated at -93 mV in the periplasmic fraction and -72 mV in the cytoplasmic fraction were not identified.

3.6.2 Inhibitor Studies

The reduction of cytochromes b and c in extracts from aerobically-grown cells and anaerobic induced cells was inhibited by both HQNO and Antimycin A. The site of inhibition appeared to be prior to the cytochromes, since the rate of reduction of both b and c-type cytochromes was apparently the same in the absence and presence of inhibitor. The thiol-group reagent PCMBS appeared to inhibit the reduction of cytochrome c more than cytochrome b in aerobic cells, indicating that it was active at a site, probably an FeS protein, in between the b and c-type cytochromes, i.e. cyt. b FeS cyt. c.

The inhibitor studies using FDH are rather more difficult to interpret in that the results suggest the involvement of a low-potential cytochrome b which was not detected spectrally. Assuming that PMS is interacting with a flavin site, then PCMBS and Antimycin A inhibit the formate dehydrogenase enzyme while HQNO may interact with the enzyme or a component between it and the cytochromes. It is not known from these results if MB and cytochrome c couple with the

aerobic or anaerobic respiratory system. More information would have been obtained from this study by (a) solubilisation of the formate dehydrogenase and repetition of the experiments to look for loss of any activities such as MB and cytochrome c reduction (b) purification of the formate dehydrogenase and repetition of the experiments with proposed physiological electron acceptors, e.g. flavocytochrome c (Chapter 5 and Section 6.2).

CHAPTER 4.

PURIFICATION OF CYTOCHROMES

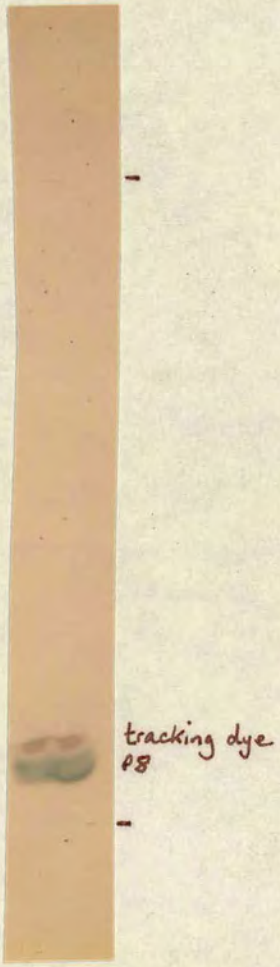
4.1 Partial Purification and Characterisation of Peak 8 (Ion Exchange)

4.1.1 Purification and Molecular Weight Determination

In crude preparations Peak 8 from ion exchange chromatography of soluble extracts from S. putrefaciens appeared to be a major low-potential cytochrome and to be formate-reducible and TMAO-oxidisable (Section 3.1). On this basis it was selected for further purification and analysis. Molecular weight determinations of ion exchange fractions of Peak 8 by SDS-PAGE revealed that it either behaved anomalously or was free haem, since cytochrome staining resulted in a single band of peroxidase activity at or near the dyefront (Plate 4.1). The cytochrome exhibited the typical spectra of a low-spin c-type cytochrome (Fig. 4.7) and of haem c when complexed with pyridine (Fig. 4.10), so it was assumed at this stage that it was a cytochrome behaving in an anomalous manner on SDS-PAGE gels.

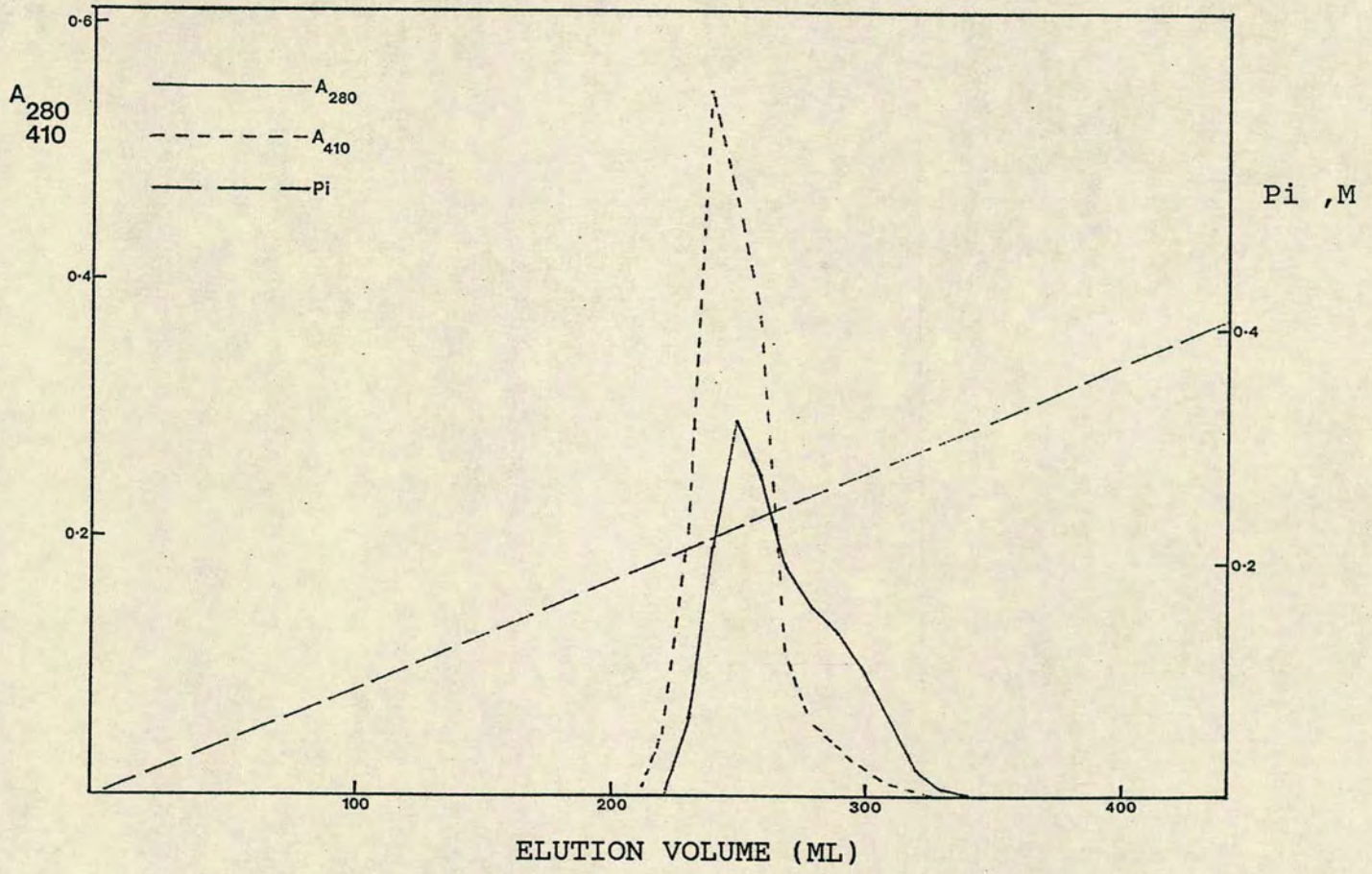
Fractions representing about 95% of the cytochrome from ion exchange chromatography (Section 3.3) were pooled and applied directly to a hydroxyapatite column. In different runs the cytochrome was consistently well resolved from other proteins and eluted at about 220 mM phosphate (Fig. 4.1). A cut representing about 90% of the total Peak 8 cytochrome from hydroxyapatite chromatography was pooled, adjusted to 2.0 M NaCl by addition of an equal volume of T- buffer containing 4.0

Plate 4.1 Purification of Peak 8



The gel was stained first for haem (blue-green) and second for protein (purple).

Fig. 4.1 Elution profile of ion exchange Peak 8 fractions chromatographed on hydroxyapatite



M NaCl, and applied to a phenyl sepharose column. The cytochrome was eluted with a 2 M⟶0 M linear NaCl gradient in T- buffer and Peak 8 eluted at about 1.6 M NaCl (Fig. 4.2). Pooled fractions containing cytochrome were dialysed against polyethylene glycol and then desalted into T- buffer by passage through a preequilibrated Sephadex G25 column. Pooled cytochromes were analysed by SDS-PAGE at each chromatographic step to monitor the progress of purification (Plate 4.1). The purified cytochrome sample used for the analyses described in this section contained only one detectable haem band which was revealed at or near the dyefront by cytochrome staining. Table 4.1 summarises the purification procedure and gives typical yields of the cytochrome, which had a purity index α_{red}/A_{280} of 1.50 (Table 4.2) and a haem content of 19.1 nmol ml^{-1} by the pyridine haemochrome method (Section 4.1.4).

The molecular weight of the cytochrome was determined by gel permeation HPLC and by standard gel permeation chromatography. The relative retention time of Peak 8 on the HPLC column was consistent with a major protein of 32 000 d (Fig. 4.3 and 4.4). Standard gel permeation chromatography revealed a band at 34 000 d which was identified as the cytochrome by absorbance at 410 nm (Figs. 4.5 and 4.6).

Fig. 4.2 Elution profile of hydroxyapatite Peak 8 fractions chromatographed on phenyl sepharose

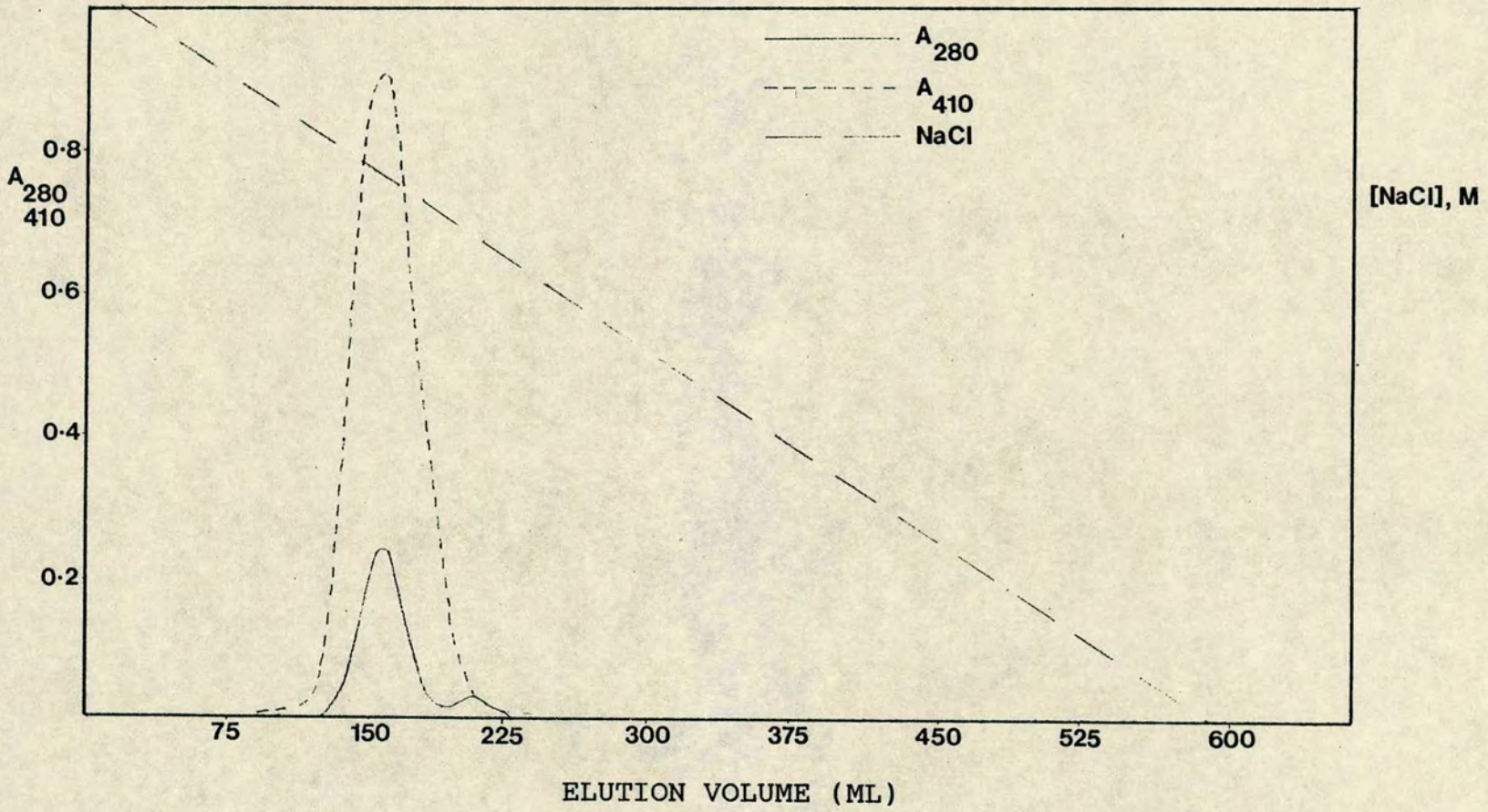


Table 4.1. Purification procedure and yields of Peak 8 from S. putrefaciens

Step	Fraction	Protein, mg (%)	Haem, nmol (%)	nmol mg^{-1} protein	Purification fold
0	Whole cells	283 (100)[1]	5040 (100)	17.81 (0.625)*	-
1	Periplasm	142 (50)[1]	3449 (68)	24.23 (1.25)*	1.36 (38.8)+
2	Ion exchange	3.18 (1.1)[2]	177 (3.5)	55.66	3.13 (89.1)+
3	Hydroxyapatite	0.61 (0.61)[2]	128 (2.5)	210	11.8 (336)+
4	Phenyl sepharose	0.405 (0.14)[3]	115 (2.3)	283	16.5 (453)+

[1]By BioRad with bovine serum albumin as standard.

[2]By A_{280} (= 1 $cm^{-1}.ml^{-1}.mg\ protein^{-1}$)

[3]By quantitative amino acid analysis.

*nmol Peak 8 haem mg $protein^{-1}$)
) assuming ion exchange fraction = 100% Peak 8 haem.
+For Peak 8 fraction only)

Fig. 4.3 HPLC of Peak 8 at 280 nm and 405 nm detection

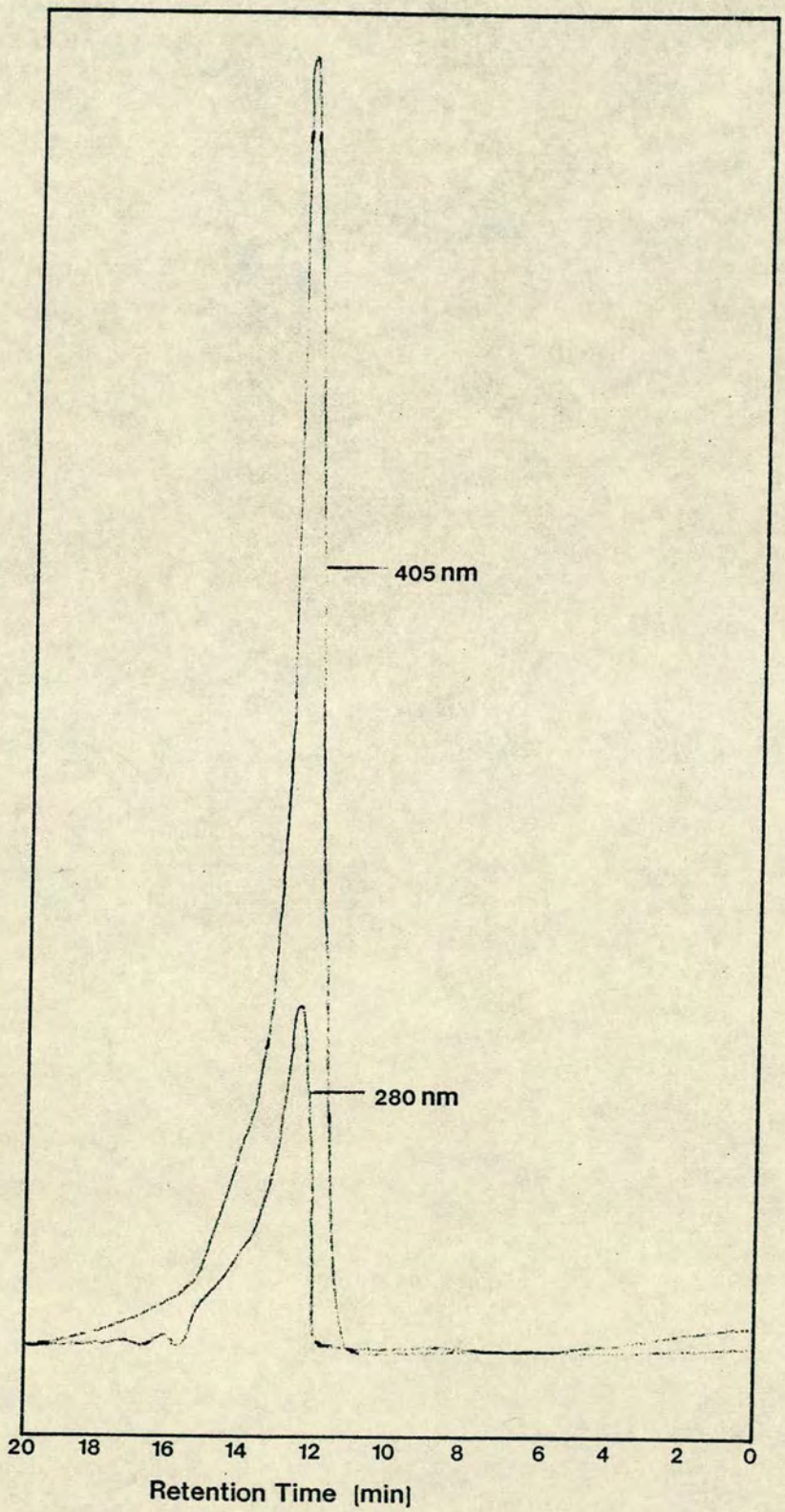
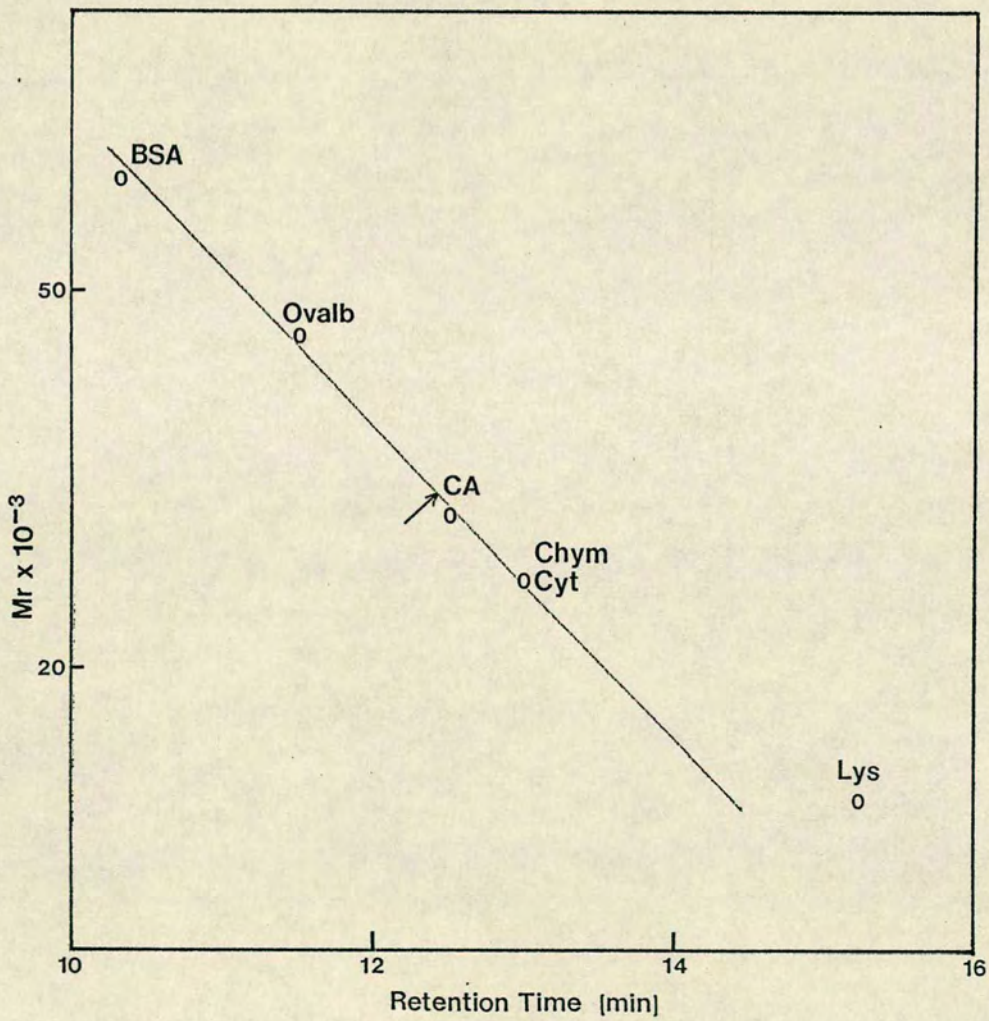


Fig. 4.4 Relative molecular weight determination of Peak 8 by HPLC



BSA: bovine serum albumin Ovalb: ovalbumin CA: carbonic anhydrase Chym: chymotrypsin Cyt: cytochrome c dimer Lys; lysozyme

Fig. 4.5 Gel filtration chromatography of Peak 8

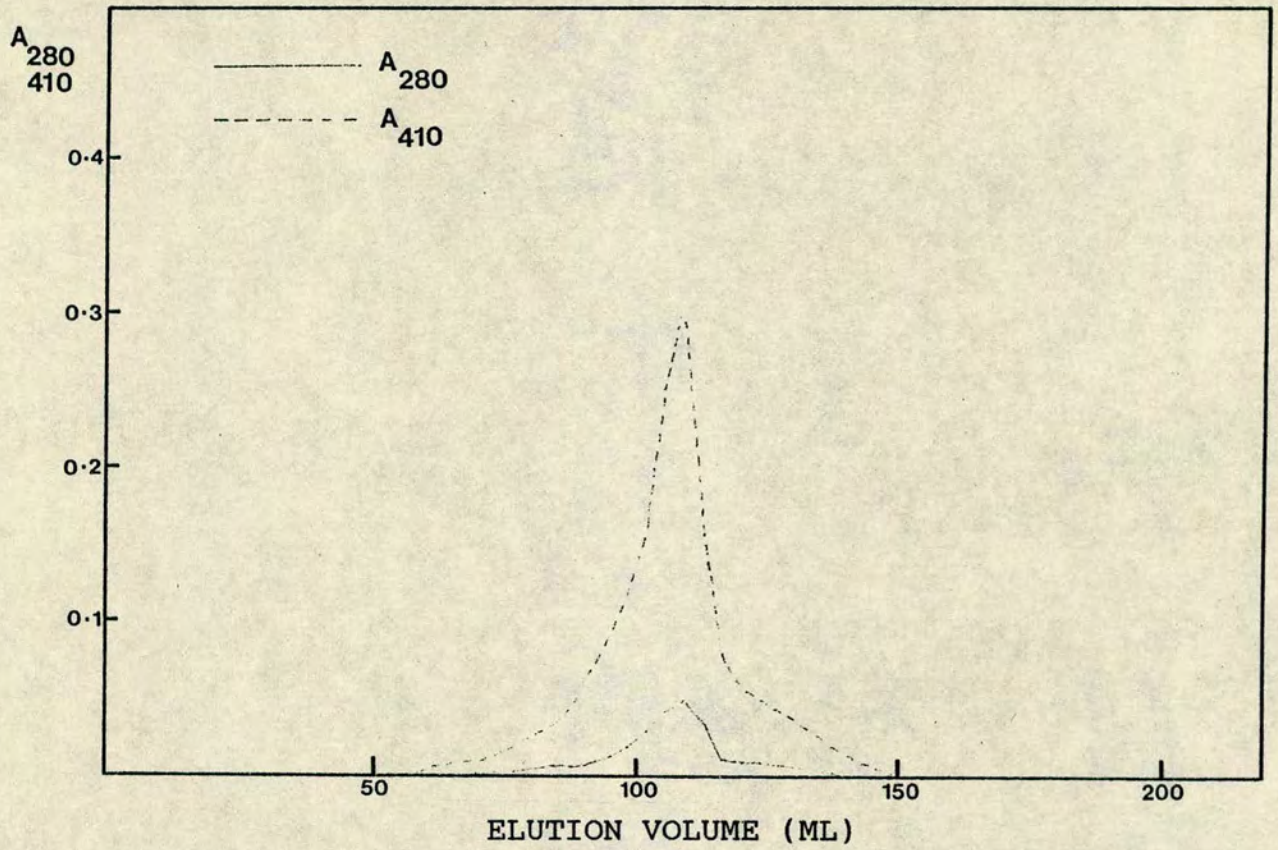
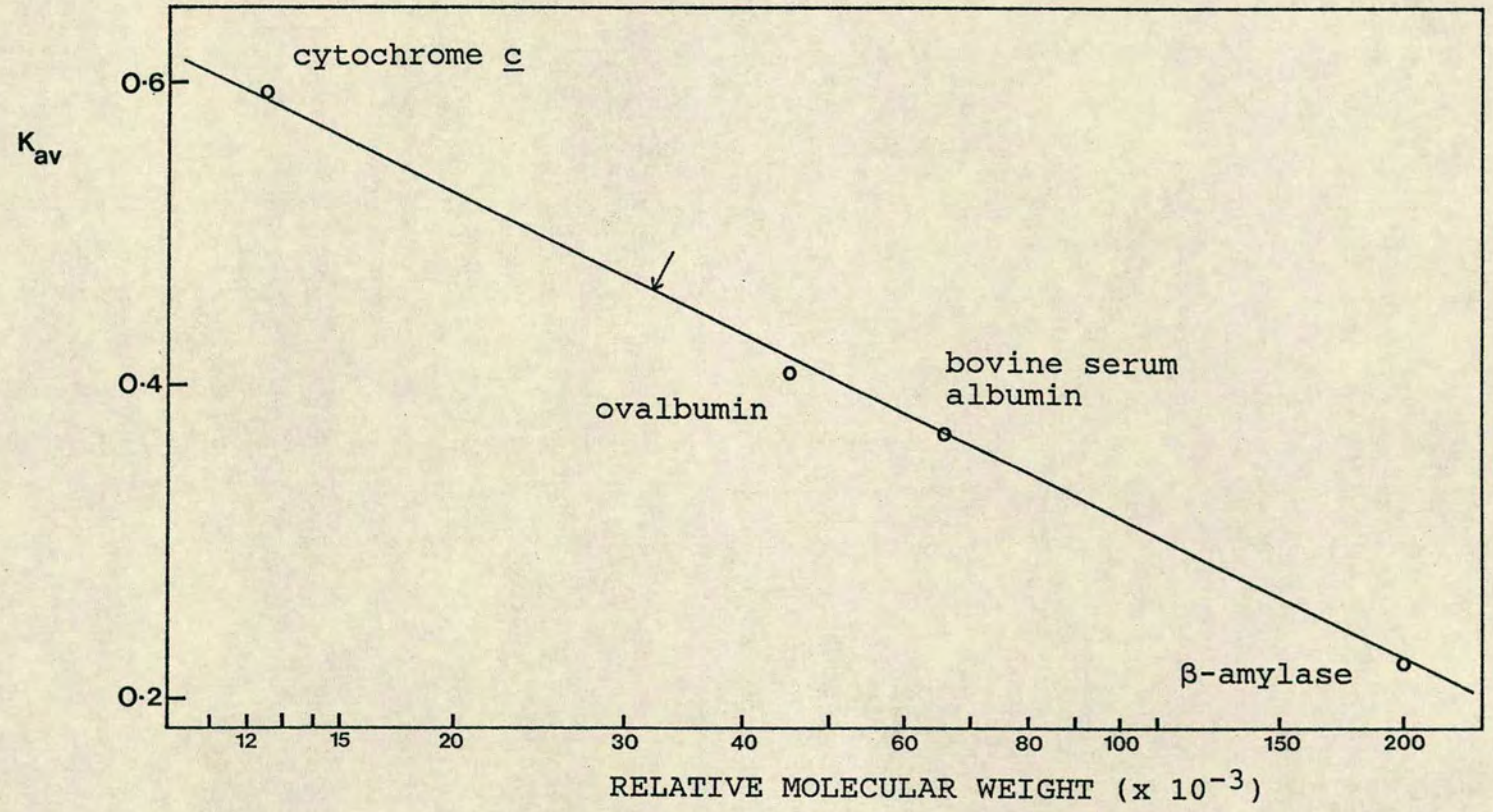


Fig. 4.6 Relative molecular weight determination of Peak 8 by gel filtration chromatography



4.1.2 Optical Spectra

The purified cytochrome solution, which was fully oxidised as prepared, was diluted 100-fold for spectra. The cytochrome was reduced by addition of solid dithionite until no further spectral changes were observed. The absolute reduced and oxidised spectra of the cytochrome are shown in Fig. 4.7 and the reduced minus oxidised spectrum is given in Fig. 4.8. The reduced cytochrome had a symmetrical α-peak at 552 nm, β-peak at 523 nm and Soret band at 419 nm. The oxidised cytochrome had a Soret band at 409, δ-band at 354 nm and shoulders at 280 and 260 nm. The reduced minus oxidised difference spectrum showed peaks at 552 (α-peak), 523 nm (β-peak) and 420 nm (Soret peak) with troughs at 443 nm and 403 nm. No 695 nm band indicative of his-Fe-met coordination was observed (Fig. 4.9). The 695 nm band of mammalian cytochrome c is included in the figure for comparison. Numerical details are given in Table 4.2.

4.1.3 Amino Acid Analysis

The amino acid composition of purified Peak 8 was determined after 20 h hydrolysis. Tryptophan is largely destroyed by the acid hydrolysis step and was not determined spectrally due to possible interference by contaminating proteins. The amino acid composition

Fig. 4.7 Absolute reduced and oxidised spectra of Peak 8

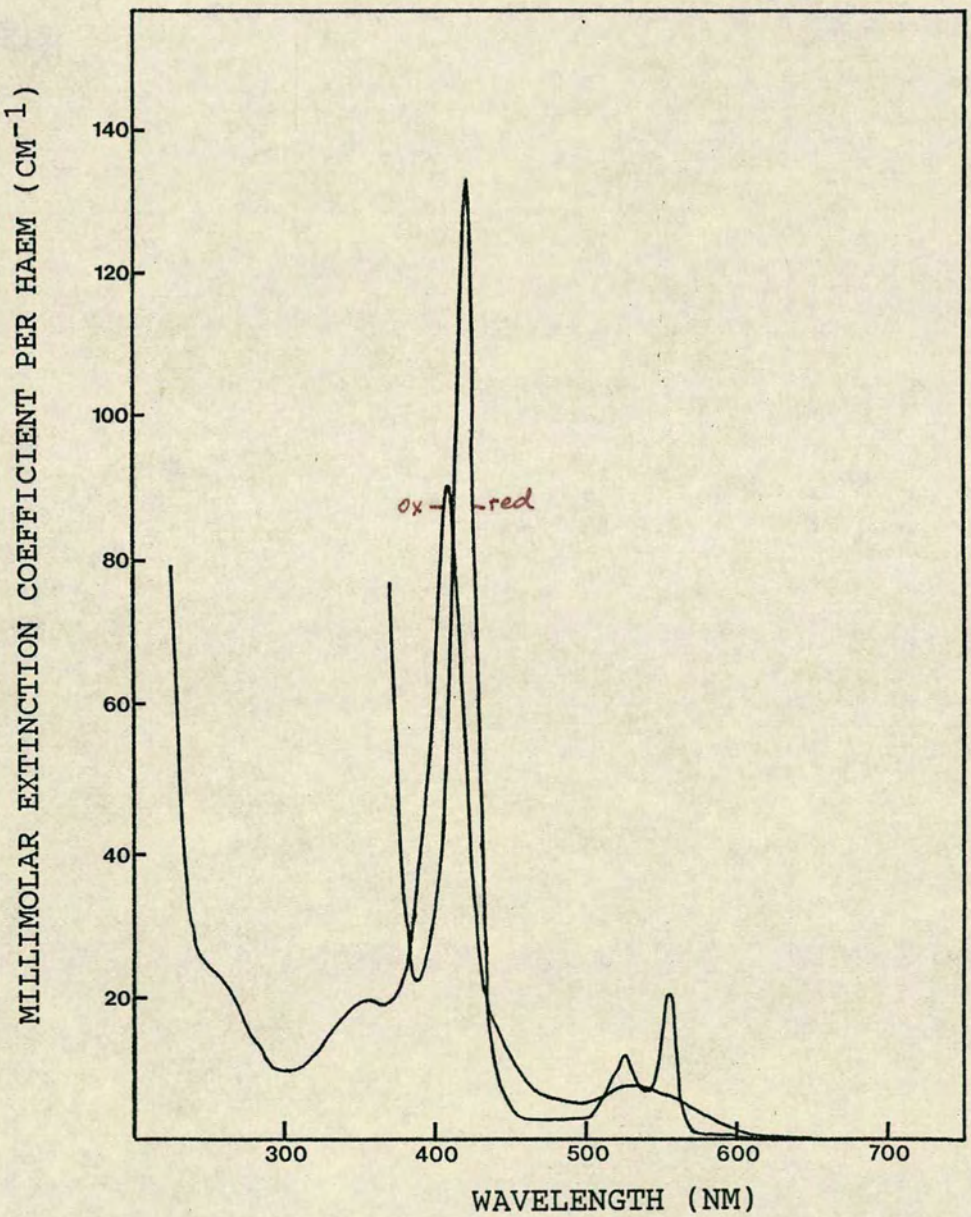


Fig. 4.8 Reduced <u>minus</u> oxidised difference spectrum of Peak 8

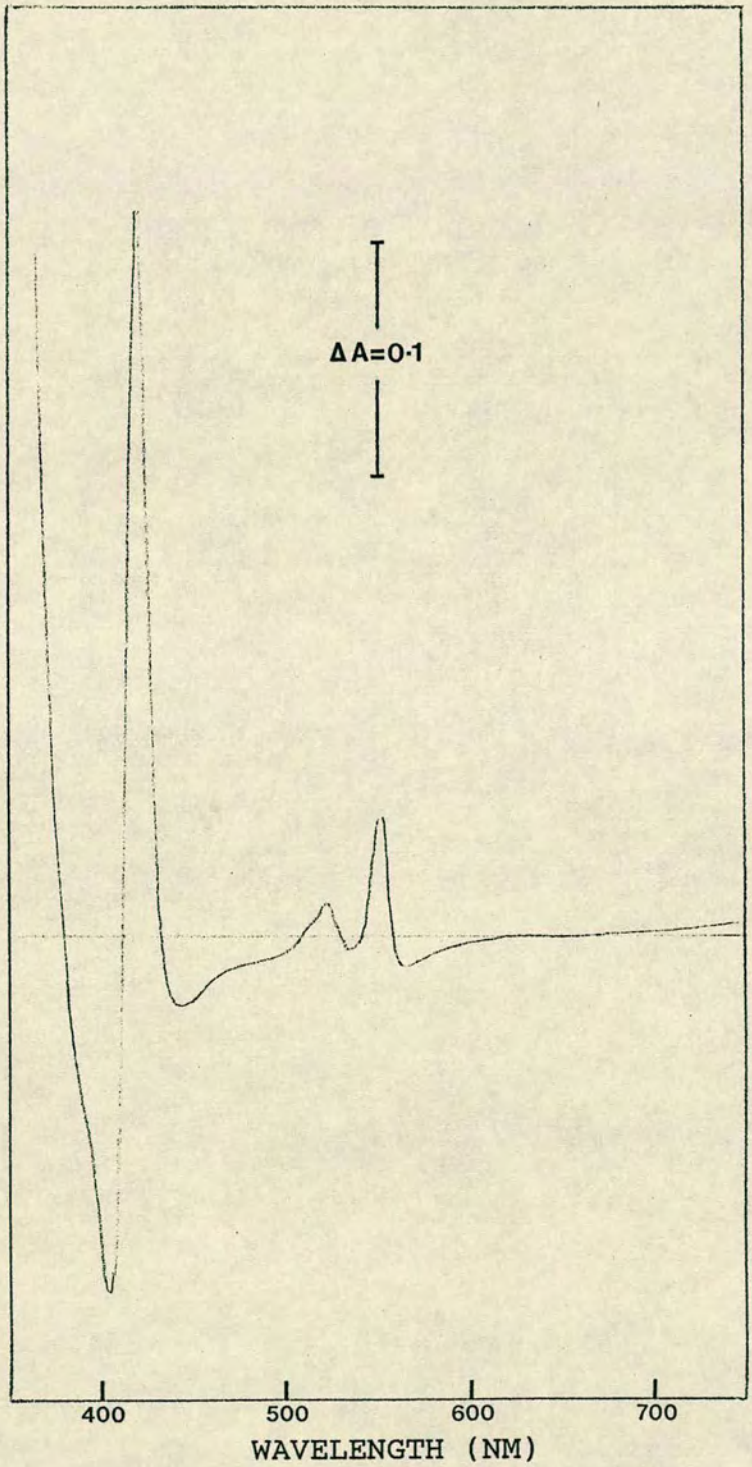
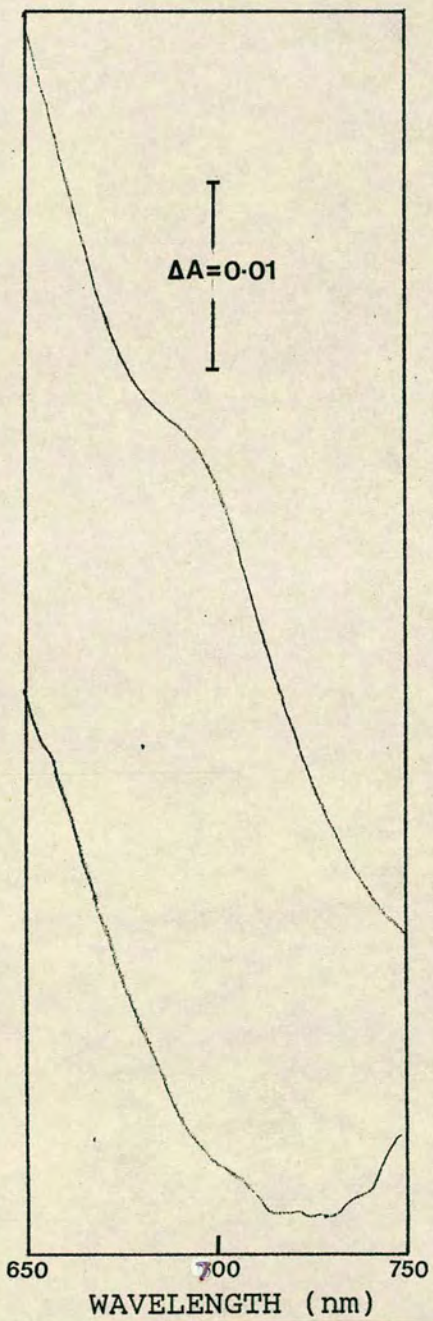


Fig. 4.9 Determination of haem ligands of Peak 8

Upper trace: horse-heart cytochrome c

Lower trace: Peak 8



Solutions normalised with respect to reduced α-peak height

Table 4.2. Optical properties of S. putrefaciens "Peak 8"

Absorption maxima (nm)			Extinction coefficients (mM^{-1} cm^{-1} $haem^{-1}$)	
Reduced	Oxidised	Band	Reduced	Oxidised
552	-	α	20.4	-
523	-	β	11.8	7.59
419	409	Soret	133.5	90.3
-	354	δ	-	19.7
-	280)		-	13.6
	) shoulders			
-	260)		-	-

$\alpha_{(red)}/A_{280_{(ox)}}$ = 1.50

$Soret_{(red)}/Soret_{(ox)}$ = 1.48

$\alpha_{(red}/\beta_{(red)}$ = 1.73

$\alpha_{(red)}/\alpha_{(ox)}$ = 3.39

Reduced minus oxidised

Peaks	Troughs
552	443
523	403
Soret 420	

of Peak 8 is given in Table 4.3. The protein content was calculated to be 67.5 μg ml^{-1}. Two features are apparent from the table. Firstly the analysis revealed 8 cysteines; haem cysteines are not quantitatively released by acid hydrolysis suggesting that cysteines were present in addition to those required for haem attachment. Also, it liberated H_2S upon treatment with mineral acid. Secondly, the cytochrome contained an unusually high number of proline residues, which tend to disrupt regularities in the structure of proteins. The cytochrome contained no tryptophan by absorbance at 280 nm, which was accounted for by tyrosine and haem.

The cytochrome contained 8 haems and the formula molecular weight was calculated to be 34 000 d.

4.1.4 Haem Determination

The determination of haem in Peak 8 was carried out as in Section 2.5. The difference spectrum of the pyridine-liganded cytochrome is shown in Fig. 4.10. It is typical of the spectra obtained for c-type cytochromes with a pair of thioether bonds between the haem group and the denatured polypeptide chain. The 550 nm α-peak is symmetrical and no other shoulders or peaks are visible which might indicate non-standard linkage of any of the haem groups or presence of other haem types in the cytochrome. The concentration of haem in the sample was calculated to be 19.1 μM, and was

Table 4.3. Amino acid analysis of Peak 8

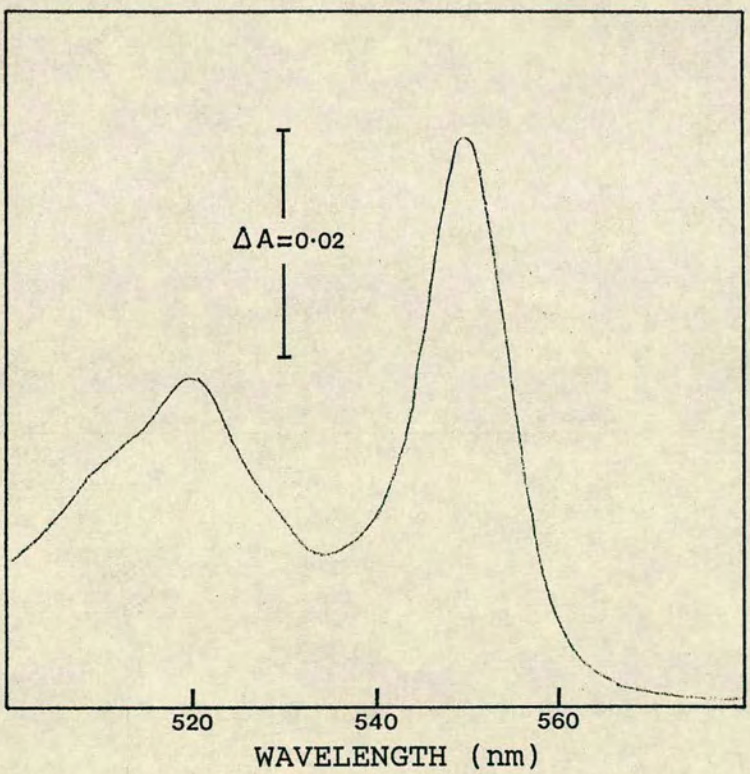
	nmol aa/ nmol haem	nmol aa/ nmol protein	Integral residues
Asp	3.98	31.8	32
Thr	1.34	10.7	11
Ser	1.01	8.1	8
Glu	3.86	30.9	31
Pro	5.38	43.0	43
Gly	2.49	19.9	20
Ala	2.98	23.8	24
Cys	1.03	8.27	8(24)
Val	0.92	7.38	7
Met	1.07	8.57	9
Ile	0.20	1.59	2
Leu	1.25	10.0	10
Tyr	0.34	2.72	3
Phe	0.74	5.92	6
His	2.3	18.4	18
Lys	1.53	12.2	12
Arg	0.44	3.52	4
			28 500

With 8 haems = 34 000 d

8 cysteines = 2(2Fe2S) or 2(4Fe4S)

Protein concentration = 67.5 $ug.ml^{-1}$

Fig. 4.10 Pyridine derivative spectrum of Peak 8



used for calculation of the molar absorbtivities of the spectral maxima of Peak 8 given in Table 4.2.

4.1.5 Redox Titrations

Peak 8 was titrated with dithionite as in Section 2.9 using 10 μM concentration of the following mediators: A2S, A26D, BV. The partially purified cytochrome showed a resistance to reduction at very low potentials (<-200 mV) as in crude preparations (Section 3.4). The redox spectrum was typical of a c-type cytochrome and had no unusual peaks or shoulders (Fig. 4.11). Analysis of the data is given in Figs. 4.12 and 4.13 which indicate the presence of at least two potentially non-equivalent haems with redox potentials of -180 mV and -300 mV.

4.1.6 Ligand Binding Properties

The ligand binding ability of Peak 8 was assayed with carbon monoxide and cyanide. CO reacted rapidly and completely with the cytochrome giving a reduced +CO minus reduced difference spectrum which was almost indistinguishable from the oxidised minus reduced difference spectrum (Fig. 4.14). Numerical details are given in Table 4.4. Peak 8 combined with cyanide in the oxidised state (Fig. 4.15) giving rise to peaks at 573, 540, 450 and 417 nm and troughs at 519 and 400

Fig. 4.11 Redox spectrum of Peak 8

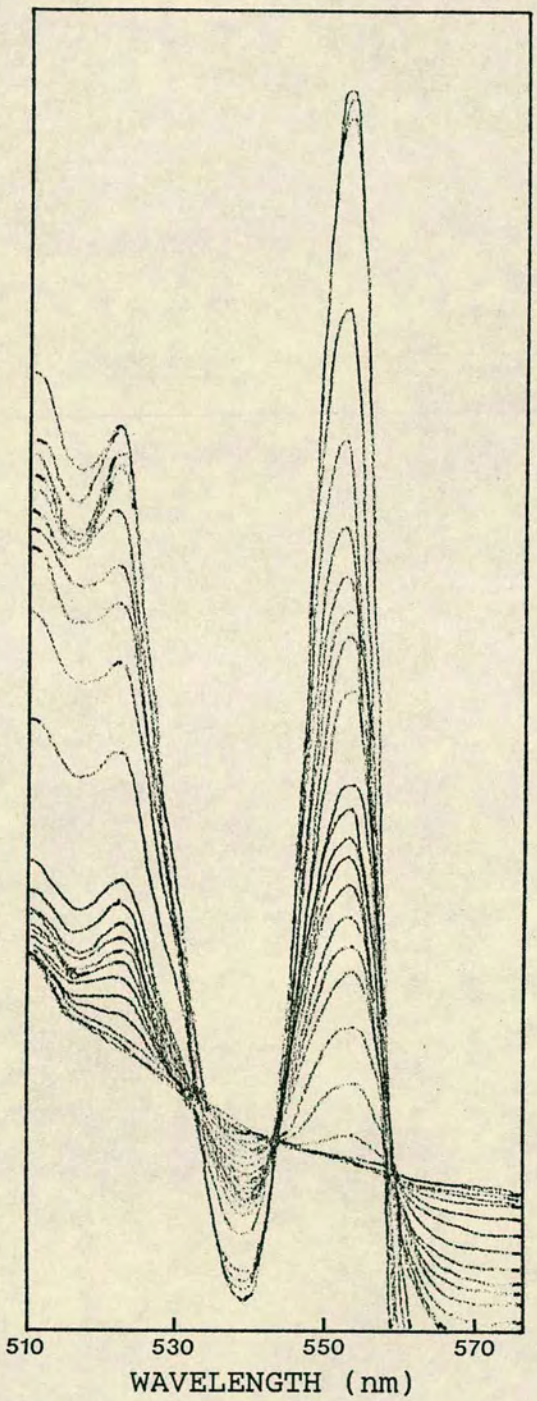


Fig. 4.12 Redox titration of Peak 8

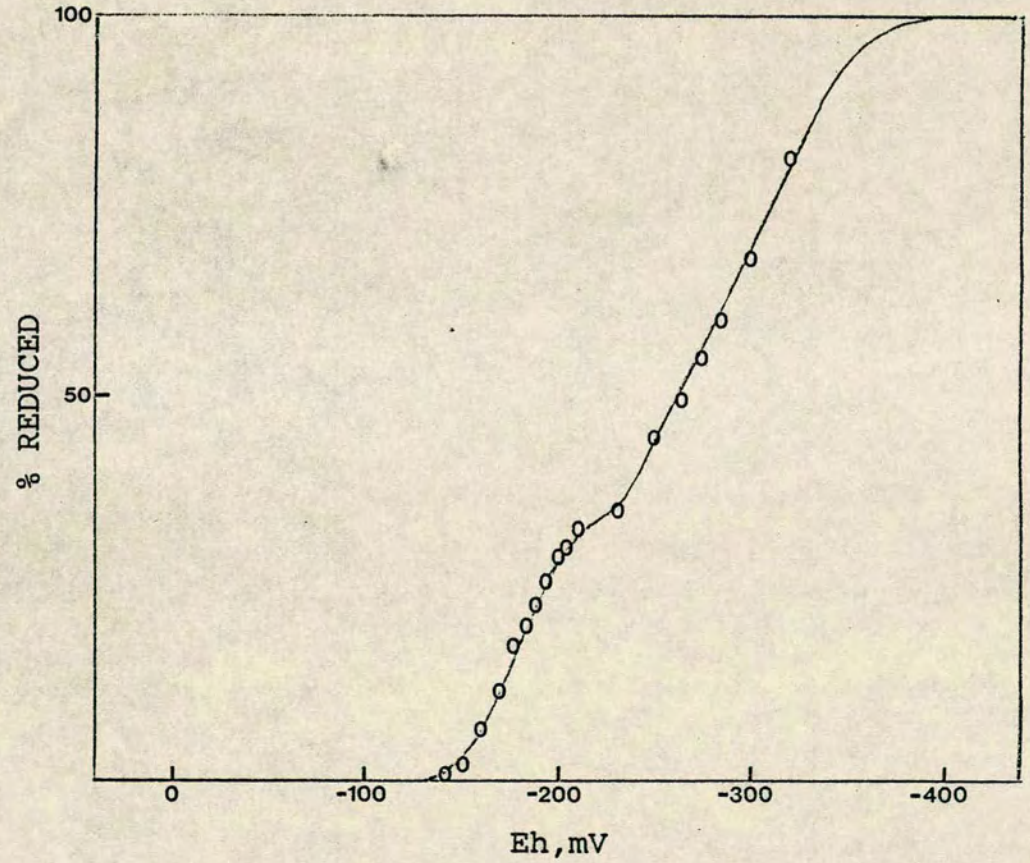


Fig. 4.13 Nernst plot of redox titration of Peak 8

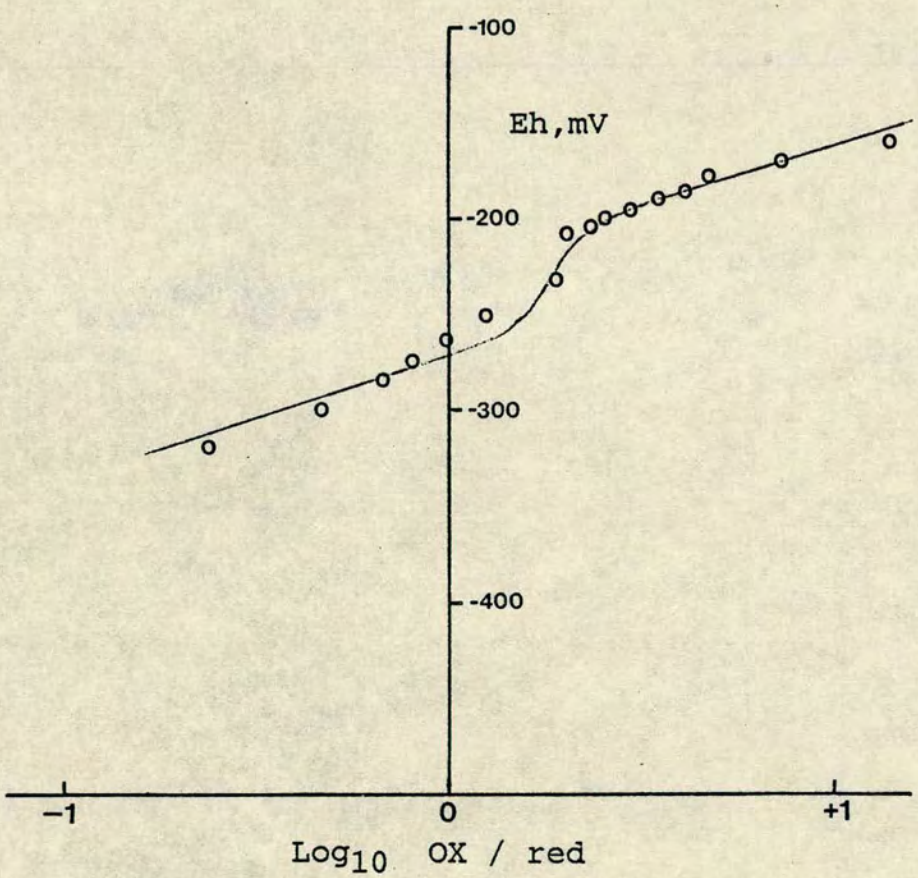


Fig. 4.14 CO-difference spectrum and oxidised minus reduced difference spectra of Peak 8

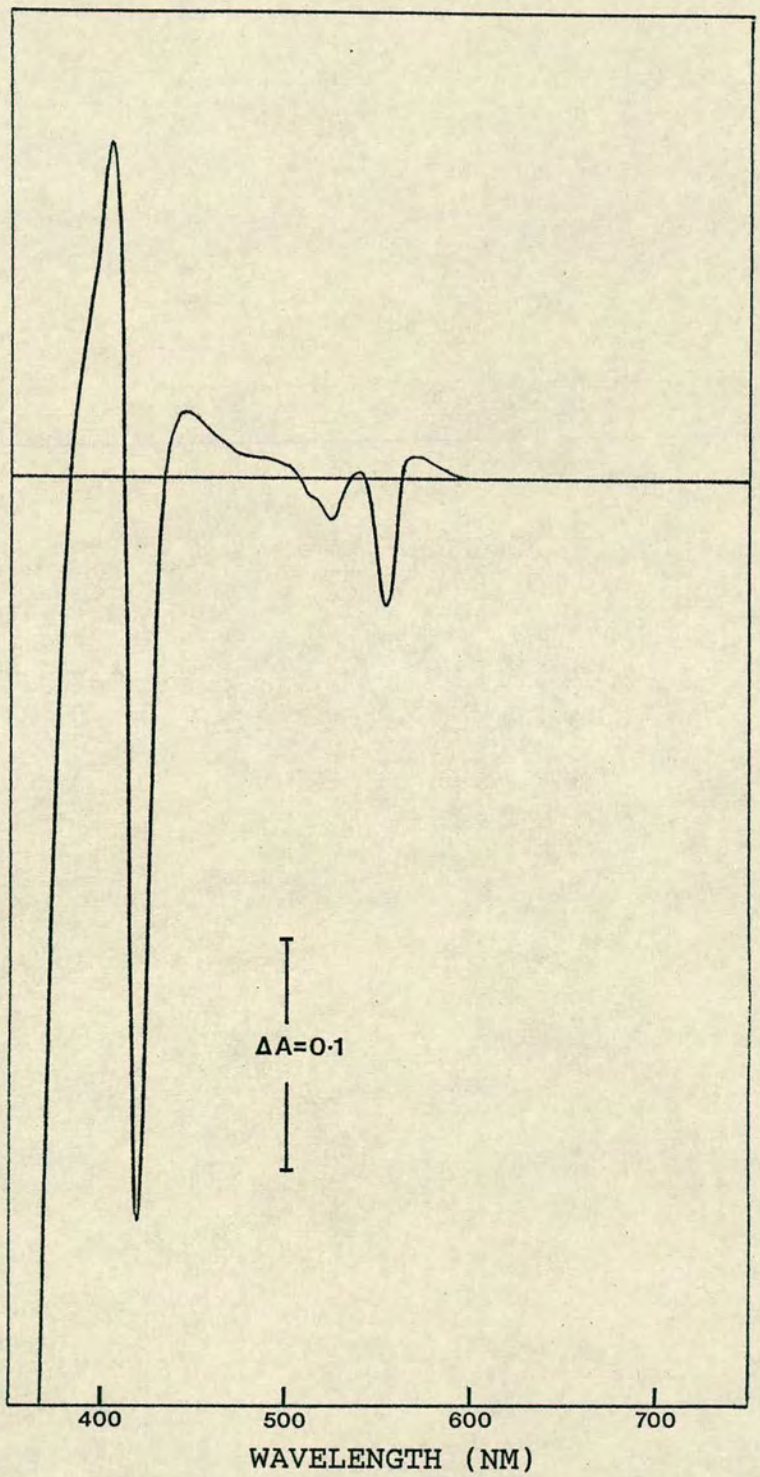


Fig. 4.15 CN^- difference spectrum of Peak 8

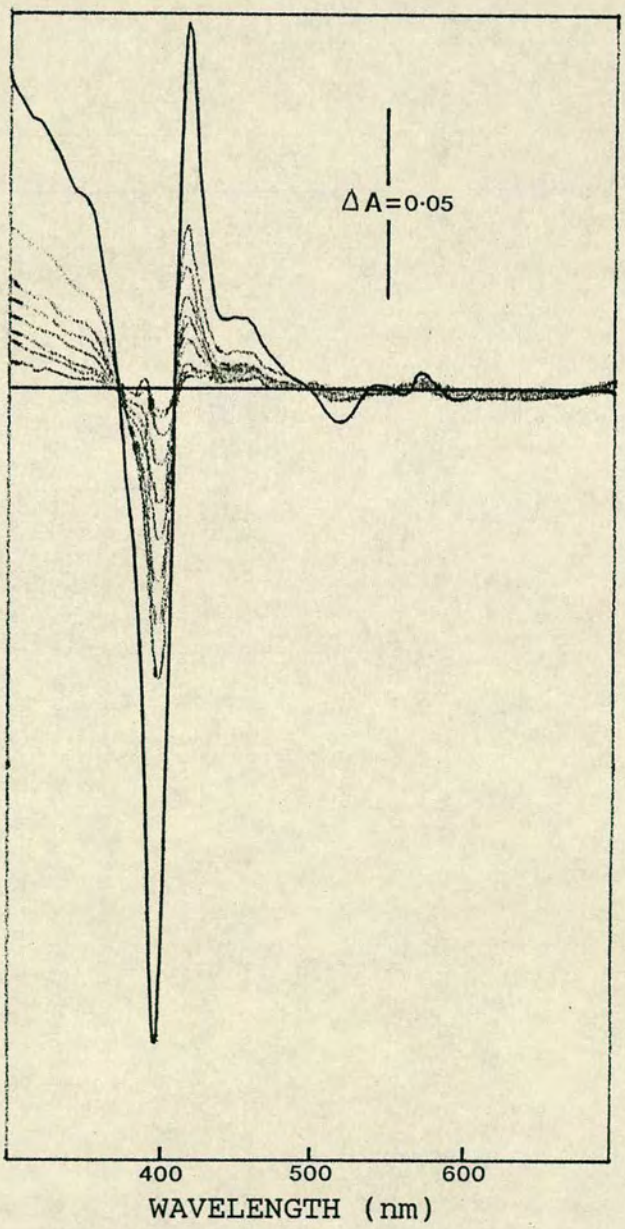


Table 4.4. Optical properties of carbonmonoxy-Peak 8

Reduced + CO minus reduced (nm)		Oxidised minus reduced (nm)	
Peaks	Troughs	Peaks	Troughs
404	552	443	552
	523	403	523
	417		420

Table 4.5. Optical properties of CN^--Peak 8 complex

Oxidised + CN^- minus oxidised	
Peaks	Troughs
573	519
540	400
450	
417	

nm (Table 4.5). Binding of CO and CN^- to the cytochrome indicated that the ligand field was weaker than that of mammalian cytochrome c, where replacement of the amino acid residue ligands requires conformational perturbation of the protein.

4.1.7 Location and Relative Content

Peak 8 was detected by SDS-PAGE/haem staining in cells grown microaerobically on TMAO, fumarate and nitrate and in cells grown under conditions of oxygen deprivation, but was difficult to detect in aerobically-grown cells. It was found principally in the periplasmic fraction, from where it was purified, but was also detectable in the cytoplasmic fraction and occasionally in membranes. The relative amount of Peak 8 in microaerobically-grown cells is shown in Section 3.3: about 15-20% of total cytochrome, similar to Peak 4 (flavocytochrome c (Chapter 5)).

4.2 Partial Purification and Characterisation of Peak 3 (Ion exchange)

4.2.1 Purification and Molecular Weight Determination

Peak 3 from ion exchange chromatography of soluble extracts of S. putrefaciens was found to be the only high potential (ascorbate-reducible) cytochrome detected in appreciable amounts by ion exchange

chromatography. It eluted at about 50 mM NaCl in a partially-reduced state (Section 3.3) and was well resolved from the other cytochrome peaks. A suitable cut representing about 90% of the cytochrome was pooled, dialysed against polyethylene glycol to a suitable volume (≈10 ml) and applied onto a hydroxyapatite column. The cytochrome, which eluted at about 200 mM phosphate (Fig. 4.16), was shown by SDS/PAGE with haemstaining to be well-separated from contaminating cytochromes and other proteins (Plate 4.2). Purification was not taken further since the results from Section 3.1 implied that high-potential cytochromes were not involved in electron transport from formate to TMAO.

The molecular weight of the partially-purified cytochrome was determined by SDS/PAGE with reference to the following marker proteins: horse-heart cytochrome c, 12 400; carbonic anhydrase, 29 000; ovalbumin, 45 000; bovine serum albumin, 67 000; and phosphorylase b, 97 000. A plot of distance migrated as a fraction of a low molecular weight dye (Pyronin Y) gave a smooth curve (Fig. 4.17), from which the relative molecular weight of the cytochrome was estimated to be 8 500 d.

4.2.2 Optical Spectra

The partially-purified cytochrome, which was

Fig. 4.16 Elution profile of ion exchange Peak 3 fractions chromatographed on hydroxyapatite

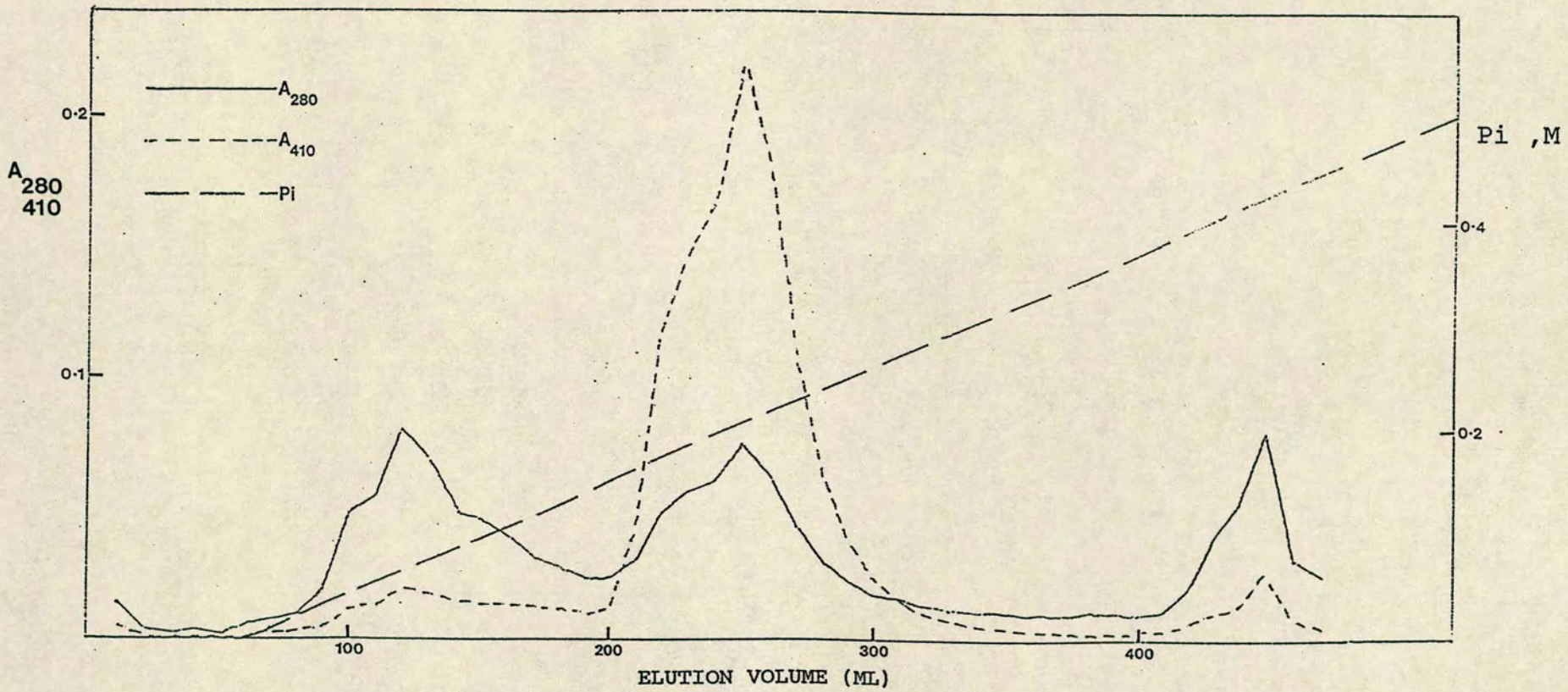
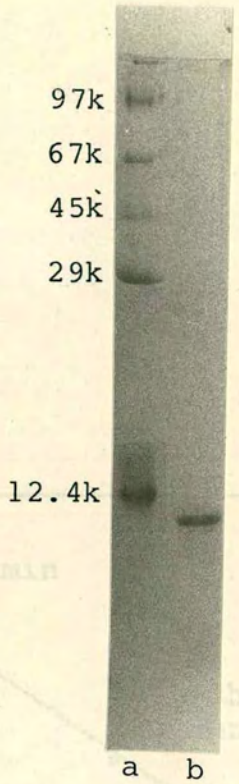


Plate 4.2 Molecular weight determination of Peak 3



15% gel stained for haem and protein

track (a): standards
track (b): peak 3

partially or fully reduced as prepared, was oxidised with 0.1% H_2O_2 and reduced by addition of solid dithionite until no further spectral changes were observed. The absolute reduced and oxidised spectra of the cytochrome are shown in Fig. 4.18. The reduced cytochrome had a symmetrical α-peak at 551 nm, β-peak at 523 nm and Soret band at 417 nm. The oxidised cytochrome had a Soret band at 409 nm. The reduced minus oxidised difference spectrum showed peaks at 551 nm, 523 nm and 418 nm with troughs at 442 nm and 404 nm.

4.2.3 Redox Titration

Titration of Peak 3 with dithionite was performed as in Section 2.9 using 10 μM final concentration of PES, PMS and DAD as mediators. In contrast with cell fractions and purified preparations of low-potential cytochromes, no experimental difficulties were encountered. The redox spectrum is given in Fig. 4.19 and is typical of a c-type cytochrome, with no evidence of unusual features such as asymmetrical α-peak, shoulders or band splitting. Analysis of the titration data (Figs. 4.20 and 4.21) revealed a single component with a midpoint potential of 216 mV.

Fig. 4.18 Absolute reduced and oxidised spectra of Peak 3

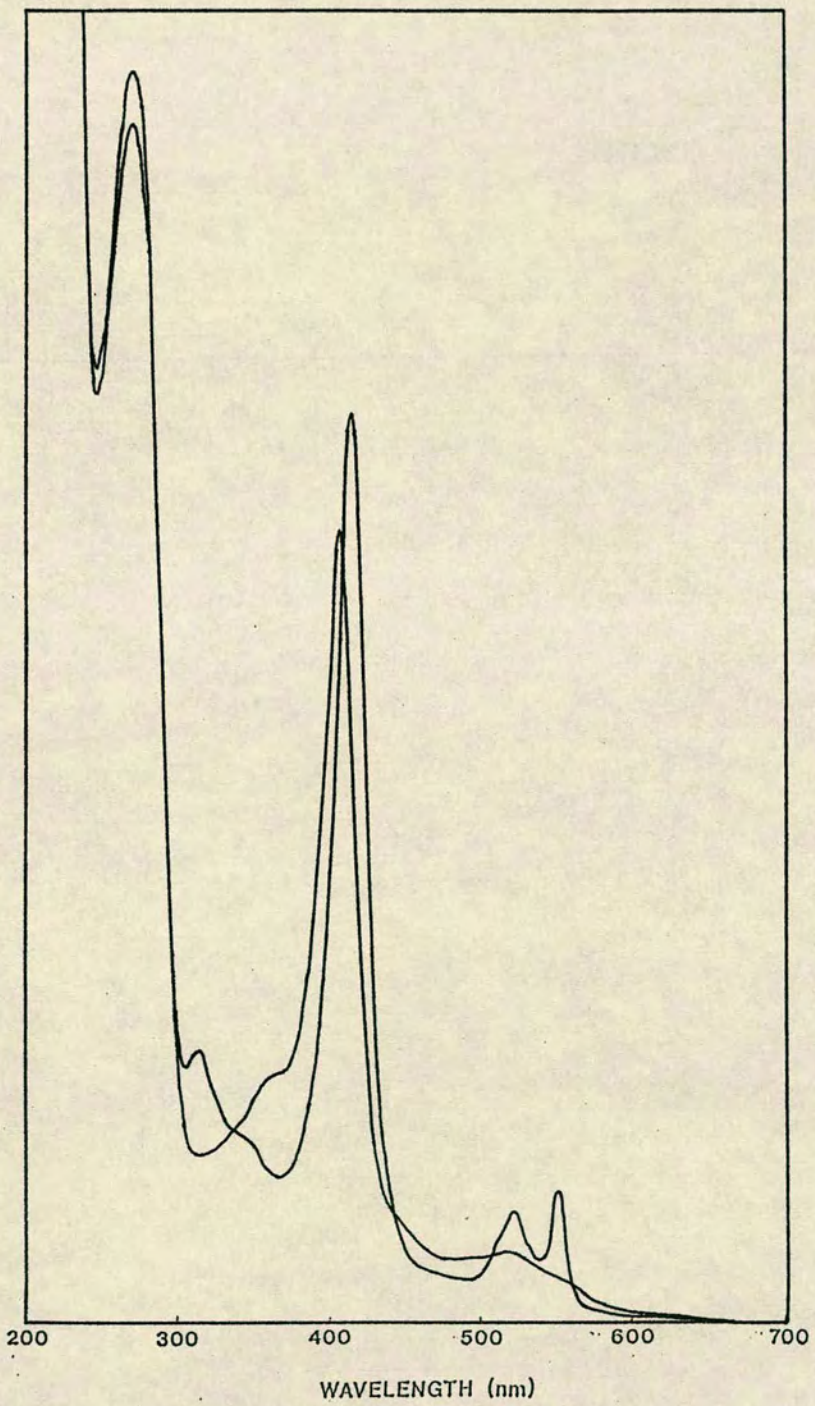


Fig. 4.19 Redox spectrum of Peak 3

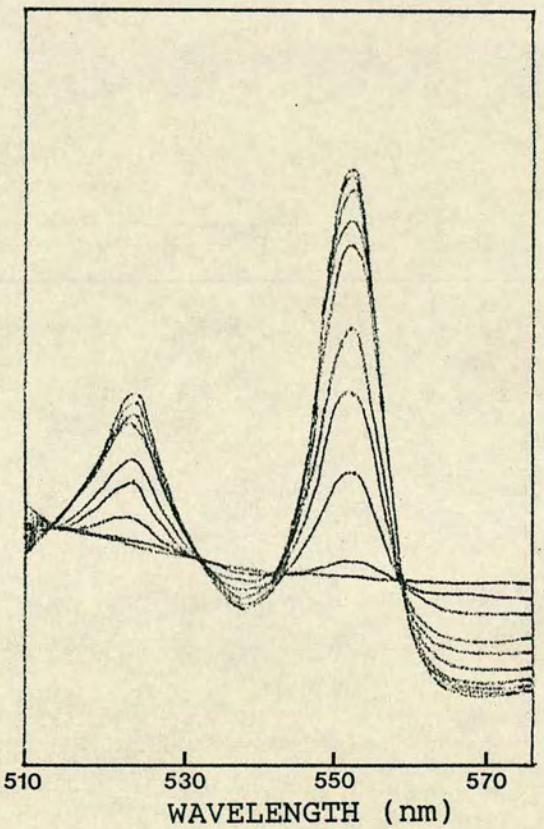


Fig. 4.20 Redox titration of Peak 3

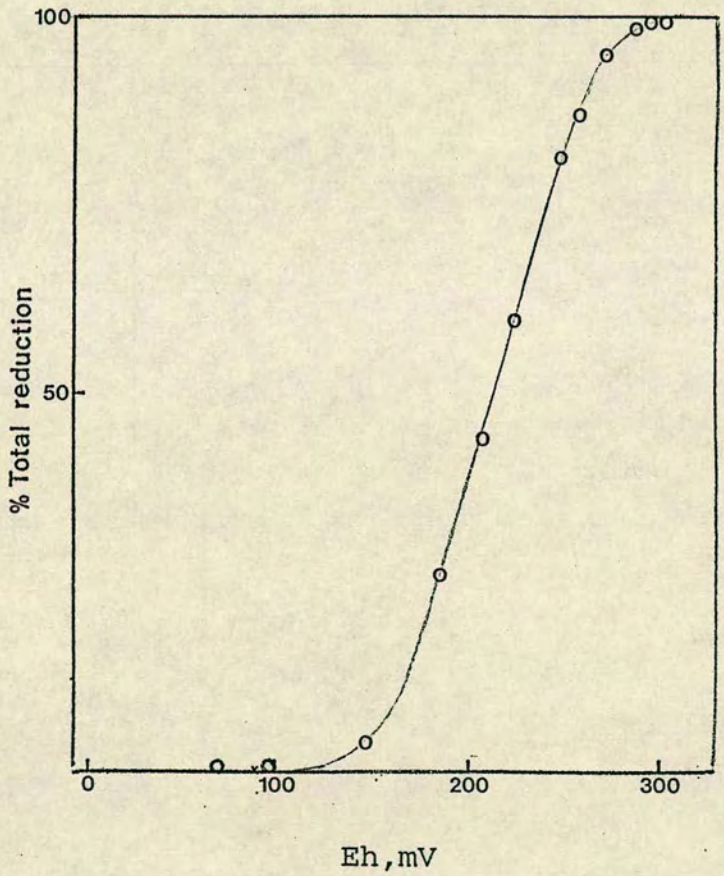
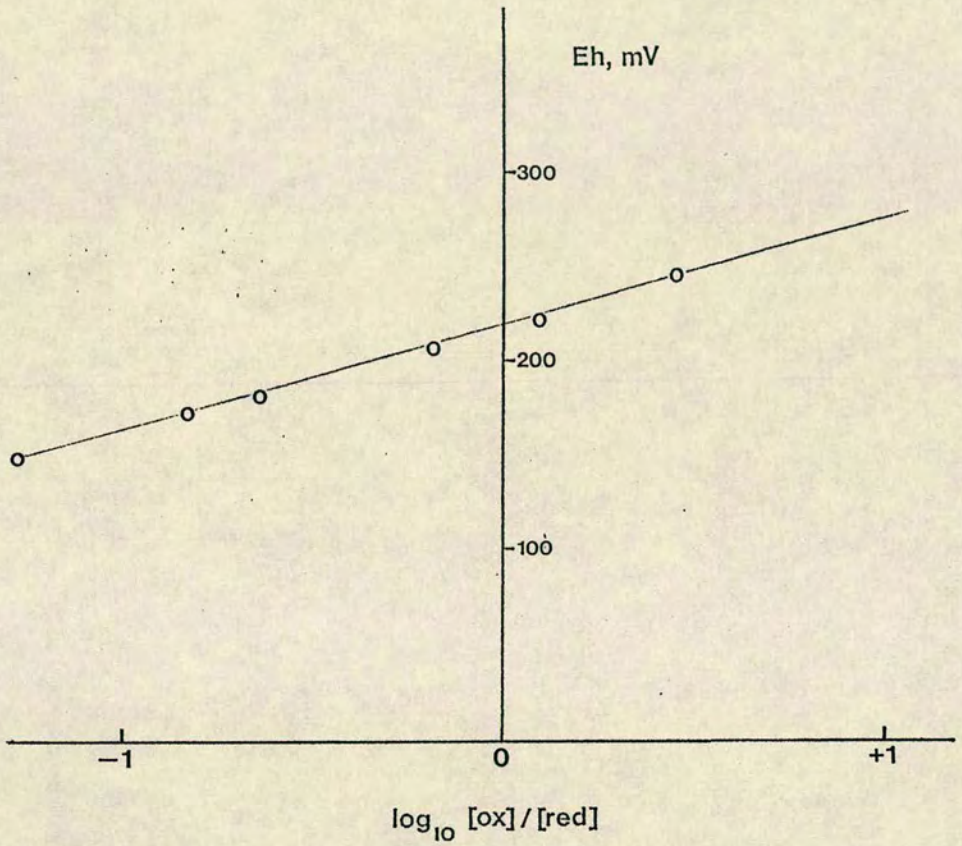


Fig. 4.21 Nernst plot of redox titration of Peak 3



4.2.4 Location and Relative Content

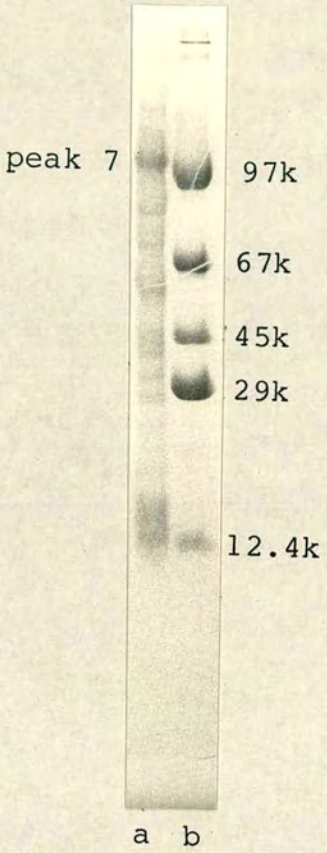
Peak 3 was a minor cytochrome component in microaerobically-grown cells where it constituted about 5-10% of the total cytochrome content. In aerobic cells it constituted a greater proportion of the total cytochrome content, estimated at about 25%. The cytochromes of aerobic cells were not analysed by prior ion exchange separation since their relative cytochrome content was prohibitively small and cells were only grown to A_{660} of 0.5 to avoid oxygen limitation. The cytochrome was found primarily in the soluble fraction, although a small proportion was detected in the particulate fraction (Section 3.2).

4.3 Partial Purification and Characterisation of Other Components

4.3.1 Peak 7 From Ion Exchange Chromatography

Peak 7 eluted from the ion exchange column in the fully-oxidised state at 200 mM NaCl (Section 3.3). The cytochrome appeared as a single smear of peroxidase activity on SDS-PAGE gels, at about 120 000 d (Plate 4.3). Purification of the cytochrome was not taken further. The spectra of Peak 7 are given in Fig. 4.22: in the reduced spectrum the α-peak is at 551 nm, the β-peak is at 523 nm, and the Soret peak is at 419 nm. In the oxidised spectrum the Soret band is at 409 nm.

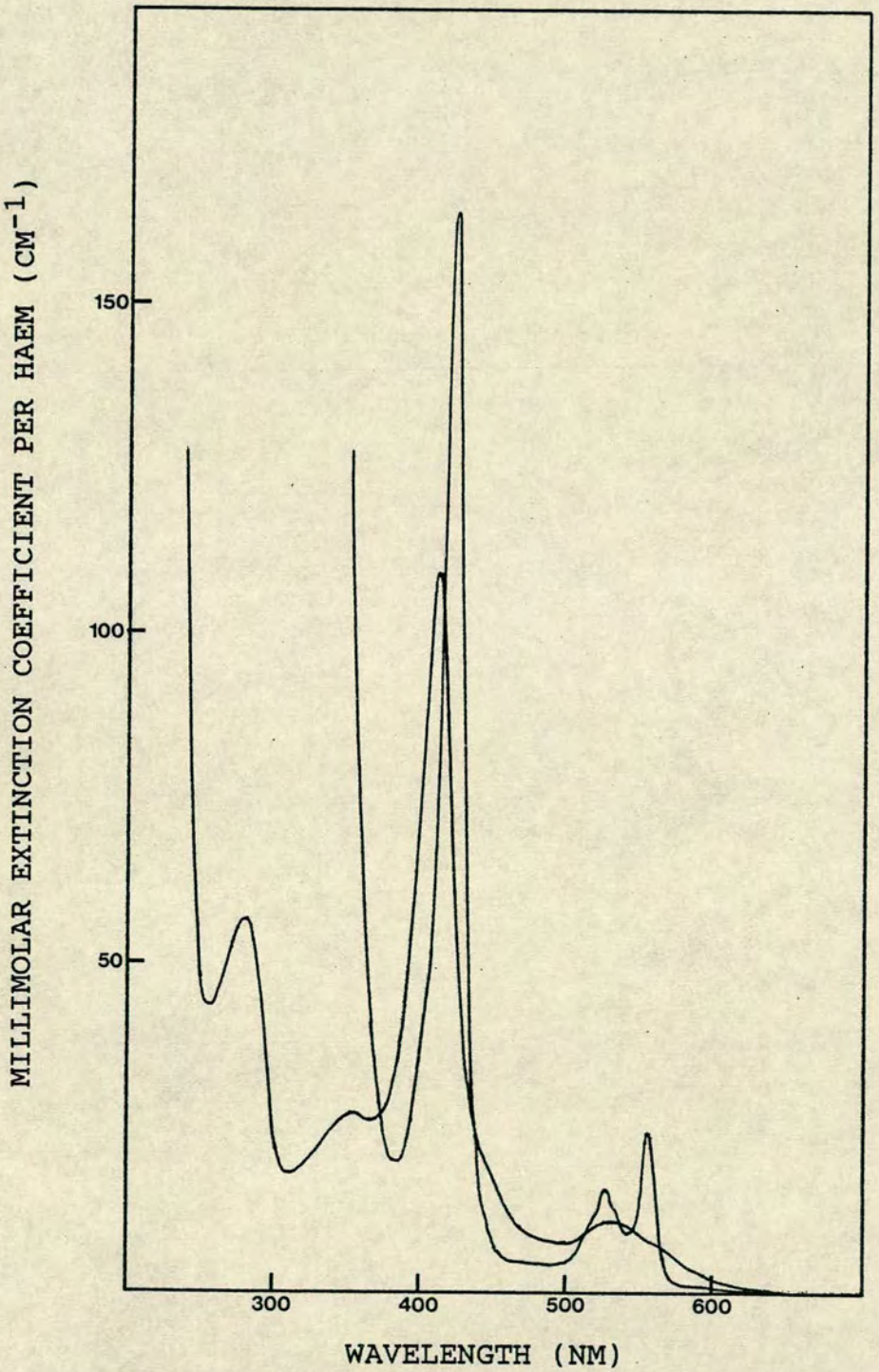
Plate 4.3 Molecular weight determination of Peak 7



10% gel stained for haem and protein

track (a): peak 7
track (b): standards

Fig. 4.22 Reduced and oxidised spectra of Peak 7



No 695 nm band was observed (results not shown) indicating bishistidyl haem coordination.

4.3.2 Redox Titration of Peak 7 From Ion Exchange

Peak 7 was titrated with dithionite (see Section 2.9) using 10 µM of the following mediators: HNQ, A2S, A26D and BV. The redox spectrum (Fig. 4.23) gave the normal shape expected for the α-band of a c-type cytochrome. Analysis of the experimental data (Figs. 4.24 and 4.25) indicated a low-potential multihaem cytochrome (probably dihaem) with redox potentials resolved at -200 mV and -286 mV. These figures are very similar to those obtained for Peak 8 (Section 4.1.5).

4.3.3 20K Cytochrome

Analysis of whole cells and cell fractions by SDS-PAGE with haemstaining (Section 3.2) revealed a band of peroxidase activity at 20 000 d in all cell types. The band was found primarily in the particulate fraction, but a proportion was noticeable in the periplasmic fraction and especially in the "EDTA-extract" (results not shown). The cytochrome did not bind to the ion exchange column, and appeared in the unbound fraction (Section 3.3). This fraction, which contained the 20 000 d cytochrome and a smear of

Fig. 4.23 Redox spectrum of Peak 7

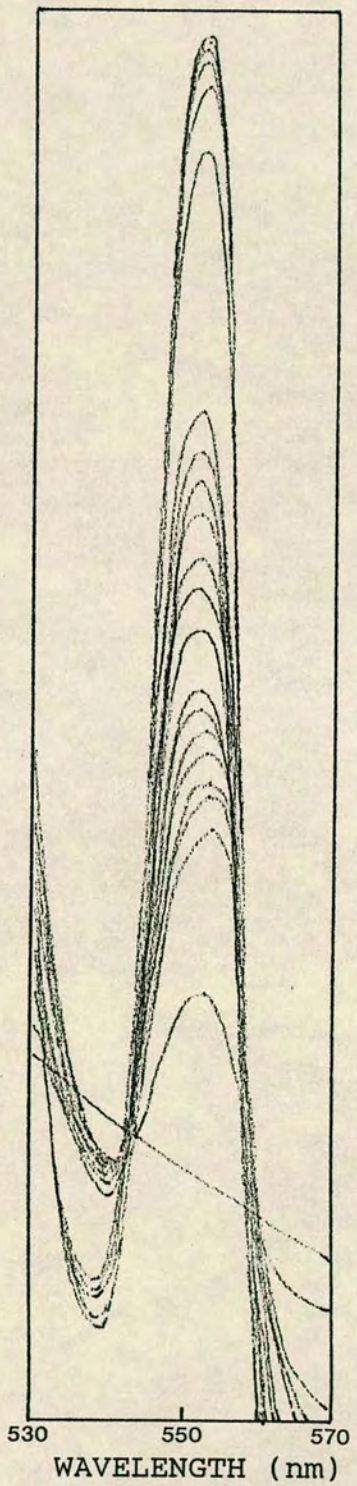


Fig. 4.24 Redox titration of Peak 7

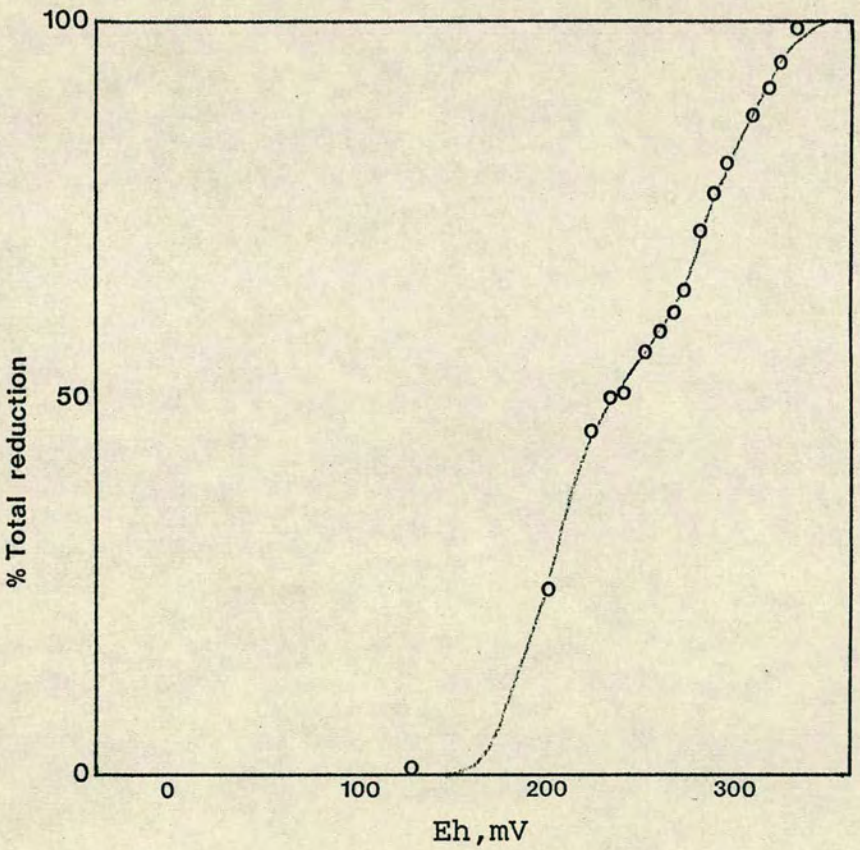
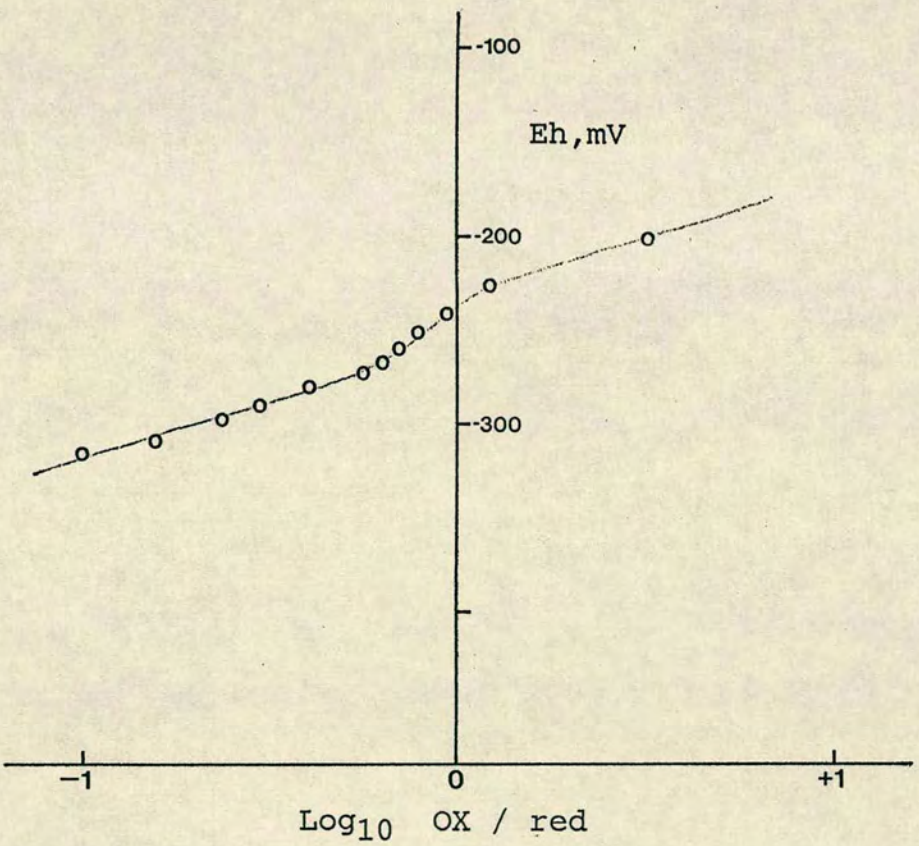


Fig. 4.25 Nernst plot of redox titration of Peak 7



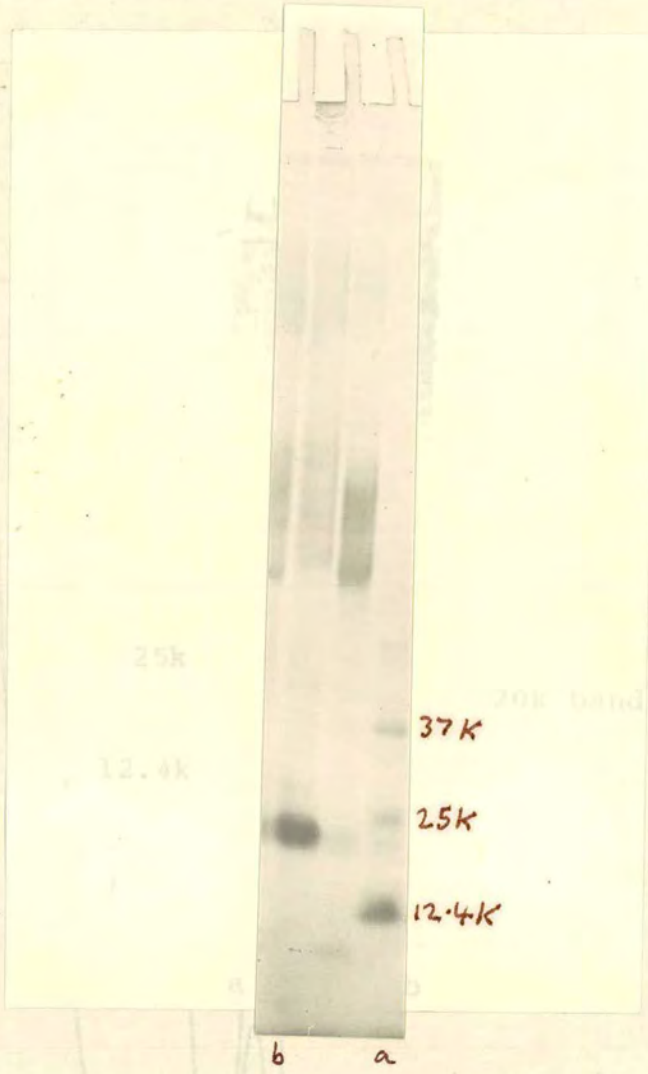
peroxidase activity similar to Peak 7 at 100-120 000 d, proved refractory to further purification due to a rapid increase in turbidity on standing. This problem was avoided by batch absorption of the cytochromes onto hydroxyapatite, and elution with 1.0 M phosphate, followed by dialysis against polyethylene glycol and T- buffer.

The resultant fraction was mixed with partially-purified cytochrome from ion exchange chromatography and applied to the phenyl sepharose column, in order to test the column for resolution of cytochromes in crude fractions. The 20 000 d cytochrome was strongly retained: it was not eluted by the decreasing NaCl gradient, but was eluted readily in a partially-reduced state under isocratic conditions using T- buffer (Fig. 5.3). No further purification was attempted. The results obtained by the steps described were analysed by SDS-PAGE (Plate 4.4). The reduced cytochrome had absorption maxima at 552, 523 and 420 nm, and the H_2O_2-oxidised cytochrome had an absorption maximum at 412 nm (Fig. 4.26). An unusual feature of the spectrum is the very small oxidised Soret band: the reason for this is not known.

4.3.4 Blue Protein

Dialysis against polyethylene glycol of pooled peaks 5, 6 and 7 from ion exchange chromatography

Plate 4.4 Molecular weight determination of the "20K band"



15% gel stained for haem

track (a): standards
track (b): 20k band

resulted in the formation of a brown precipitate. However, this readily resuspended in T- buffer and the resultant preparation was reapplied to the anion exchange column and a linear increasing NaCl gradient was applied as previously. The cytochromes eluted as expected but a blue band remained adsorbed at 500 mM NaCl. This fraction was eluted with 2 M NaCl and when analysed spectrally, was found to contain cytochrome c absorbing at 552, 523 and 420 nm with a further broad band at 620 nm (Fig. 4.27) which was bleached by dithionite. These results suggested that the fraction contained a copper protein, possibly azurin.

Further purification of the blue protein proved difficult. When cation exchange chromatography at low pH was attempted, the protein rapidly precipitated during the dialysis buffer exchange. In hydroxyapatite chromatography the blue protein bound very strongly and was not cleanly eluted. The protein required 2 M phosphate pH 8.4 for elution and even at this concentration some blue colour remained adsorbed to the column. SDS-PAGE gels revealed six protein bands (Plate 4.5) at 12 000 d, 25 000 d, 27 000 d, 36 000 d, 43 000 d and 45 000 d but it is not known which of these protein bands corresponded to the blue protein.

4.4 Discussion

With an estimated 8 haems per 32 000 d

Fig. 4.27 Oxidised spectrum of the blue protein from *S. putrefaciens*

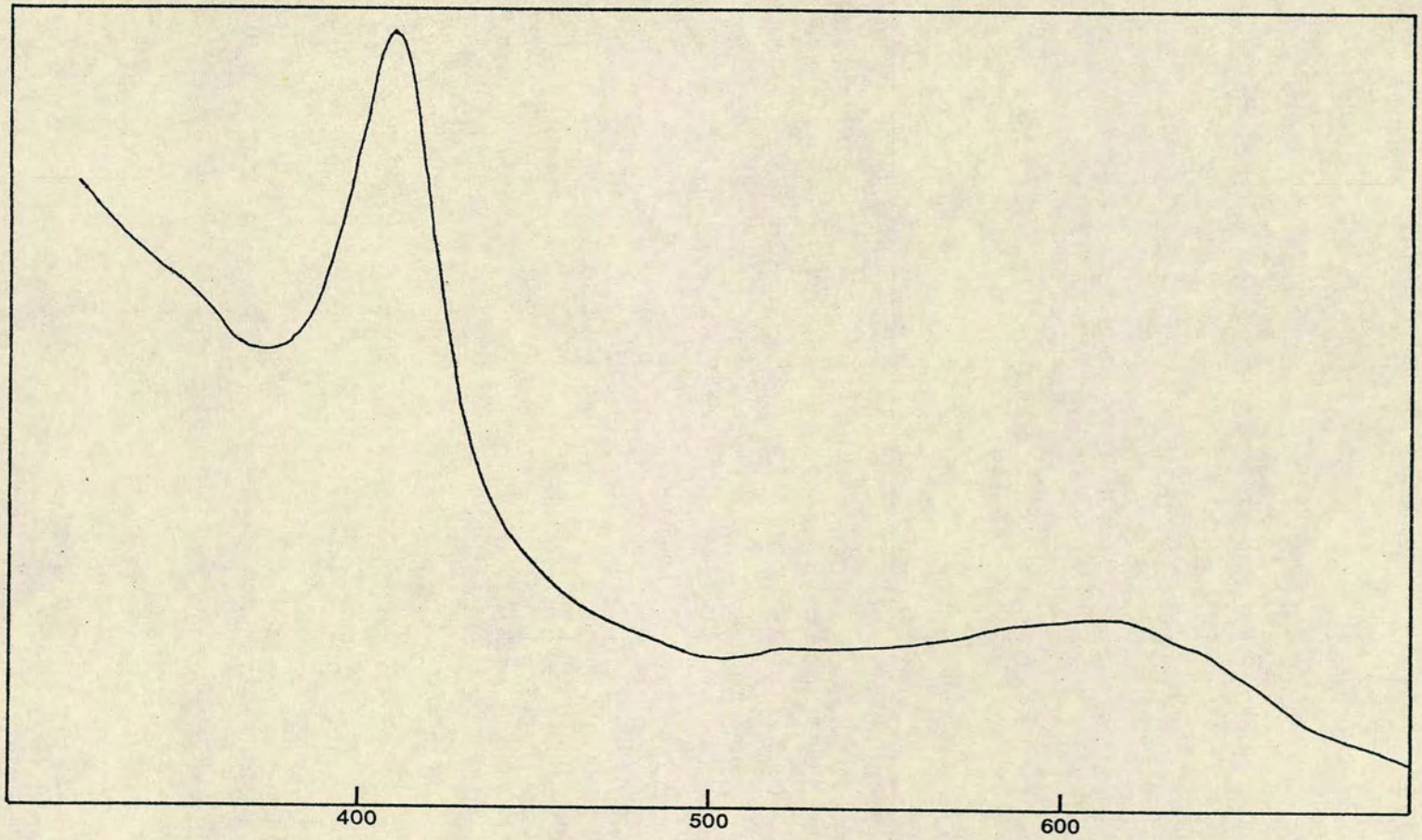
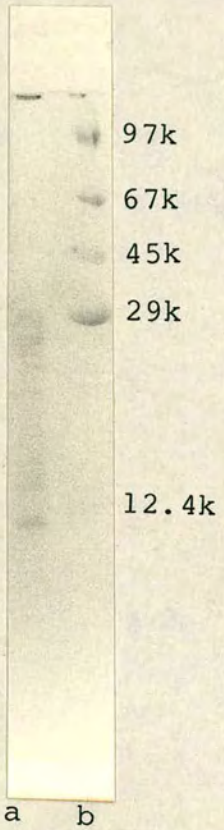


Plate 4.5 Resolution by SDS-PAGE of protein present in the blue fraction from S. putrefaciens



15% gel stained for protein

track (a): blue protein
track (b): standards

polypeptide, low redox potential, low absorbtivity at short wavelengths, small shoulder on the short-wavelength side of the reduced Soret band, bishistidyl coordination and a high $Soret_{(red)}/Soret_{(ox)}$ ratio, Peak 8 exhibited all of the distinguishing features for a Class III cytochrome (Section 1.3.3). Indeed it appears to share a lot of common features with the 26 000 d octahaem cytochrome c_3 of Desulfovibrio gigas which is reactive with hydrogenase (Bell et al., 1978), and it is suggested that a similar function is a possibility for the 32 000 d cytochrome of S. putrefaciens (Section 6.3).

The cytochrome contained non-haem binding cysteine and released H_2S on treatment with acid indicating that it contained FeS clusters each requiring 4 cysteine residues. Non-haem-binding cysteine residues are rare in cytochromes: the small Class I cytochromes of the methylotrophs Pseudomonas AM 1 (O'Keefe & Anthony, 1980) and Methylophilus methylotrophus (Cross & Anthony, 1980) both contain a free cysteine residue. There are no reports of a c-type cytochrome containing FeS clusters; acid-labile sulphide and total iron determination would provide further evidence of their presence, which could be confirmed by EPR studies.

With its low redox potentials and c_3-type spectrum, Peak 7 is superficially similar to the flavocytochrome (Peak 4: Section 5). However with a molecular weight in excess of 100 000 d on denaturing

gels it is apparently in a class of its own at present.

The results presented in this section give strong support for the presence in S. putrefaciens of the c_{551} / c_4 / c_5 / azurin group of redox components. Peak 3, with a relative molecular weight of 8 500 d, redox potential of 217 mV and α-peak at 552 nm correlates closely with c_{551}, described (Meyer & Kamen, 1982) as an 8-9 000 d monohaem monomer with redox potentials from 200-265 mV and α-peak from 551-553 nm. The "20K cytochrome" appears to be similar to c_4, which is described as a membrane-bound dihaem monomer of 20 000 d with α-peak varying from 550-552 nm, redox potential from 200-300 mV and α/β ratio from 1.2-1.3. Cytochrome c_5, a dimer of monohaem 10 000 d subunits, α-peak at 555 nm and α/β ratio of 1.4, may correspond with the 11 000 d band (Section 3.2): unfortunately no further data is available. The blue protein may correspond with azurin, although copper analysis and molecular weight determination might represent minimum criteria to substantiate this.

CHAPTER 5.

S. putrefaciens FLAVOCYTOCHROME *c*

5.1 Purification and Molecular Weight Determination

Peak 4 from ion exchange chromatography of periplasmic fractions was selected for purification as it contained a major low potential cytochrome c in soluble extracts of S. putrefaciens and was shown to be formate-reducible and TMAO-oxidisable in crude preparations. A suitable cut representing approximately 95% of the cytochrome fraction from ion exchange of cell extracts (see Section 3.3) was dialysed o/n against polyethylene glycol (PEG) and applied to a hydroxyapatite column. Elution profiles (Fig. 5.1) consistently showed good separation of the proteins by this method and only one significant haem-containing protein was found eluting at about 125 mM phosphate. A cut representing about 90% of the cytochrome was taken from hydroxyapatite chromatography, dialysed against PEG and applied to a phenyl sepharose column. The sample (10 ml) was mixed with an equal quantity of T- buffer containing 4 M NaCl for loading. The cytochrome was eluted with a 4 M-0 M linear NaCl gradient and a single peak eluted at about 1.7 M NaCl (Fig. 5.2). All fractions having an A_{410} greater than 0.1 were pooled and dialysed against PEG to 3 ml. The dialysate was desalted by gel filtration on Sephadex G25 into T- buffer and diluted to exactly 5 ml. The efficiency of the phenyl sepharose column for separating S. putrefaciens

Fig 5.1 Elution profile of ion exchange flavocytochrome c fractions chromatographed on hydroxyapatite

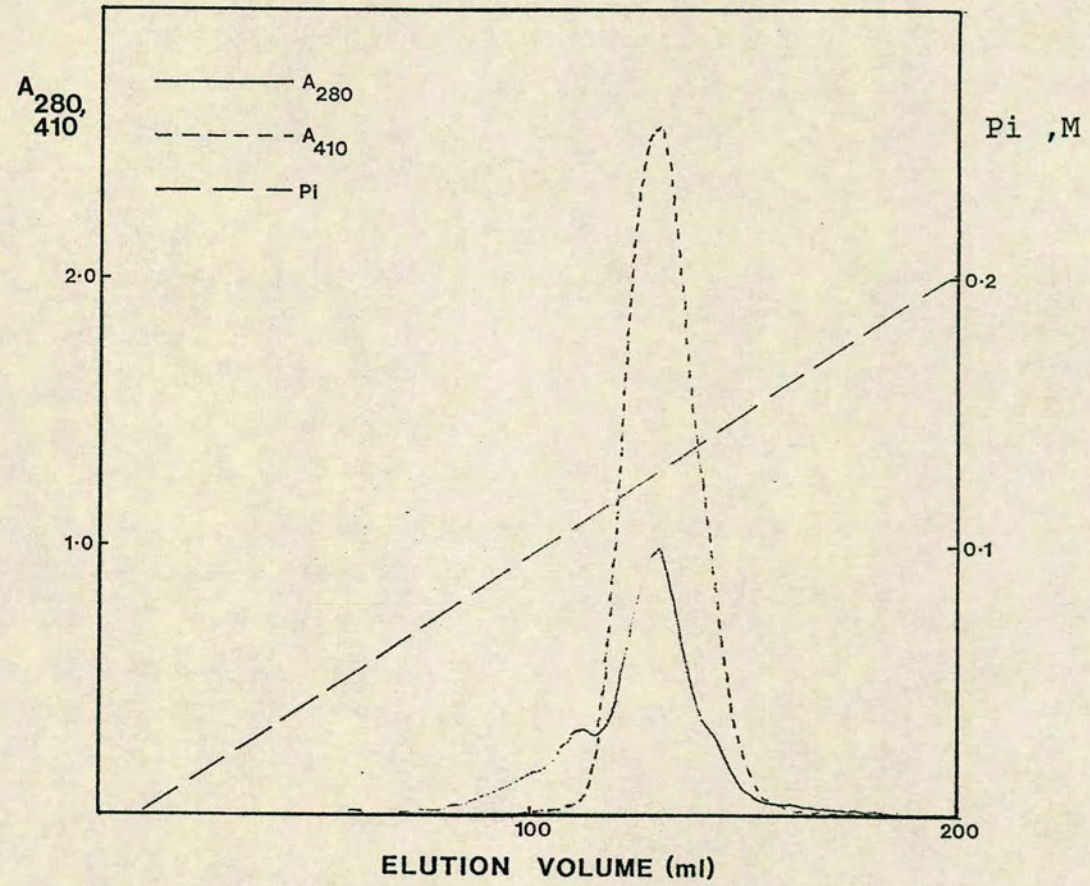
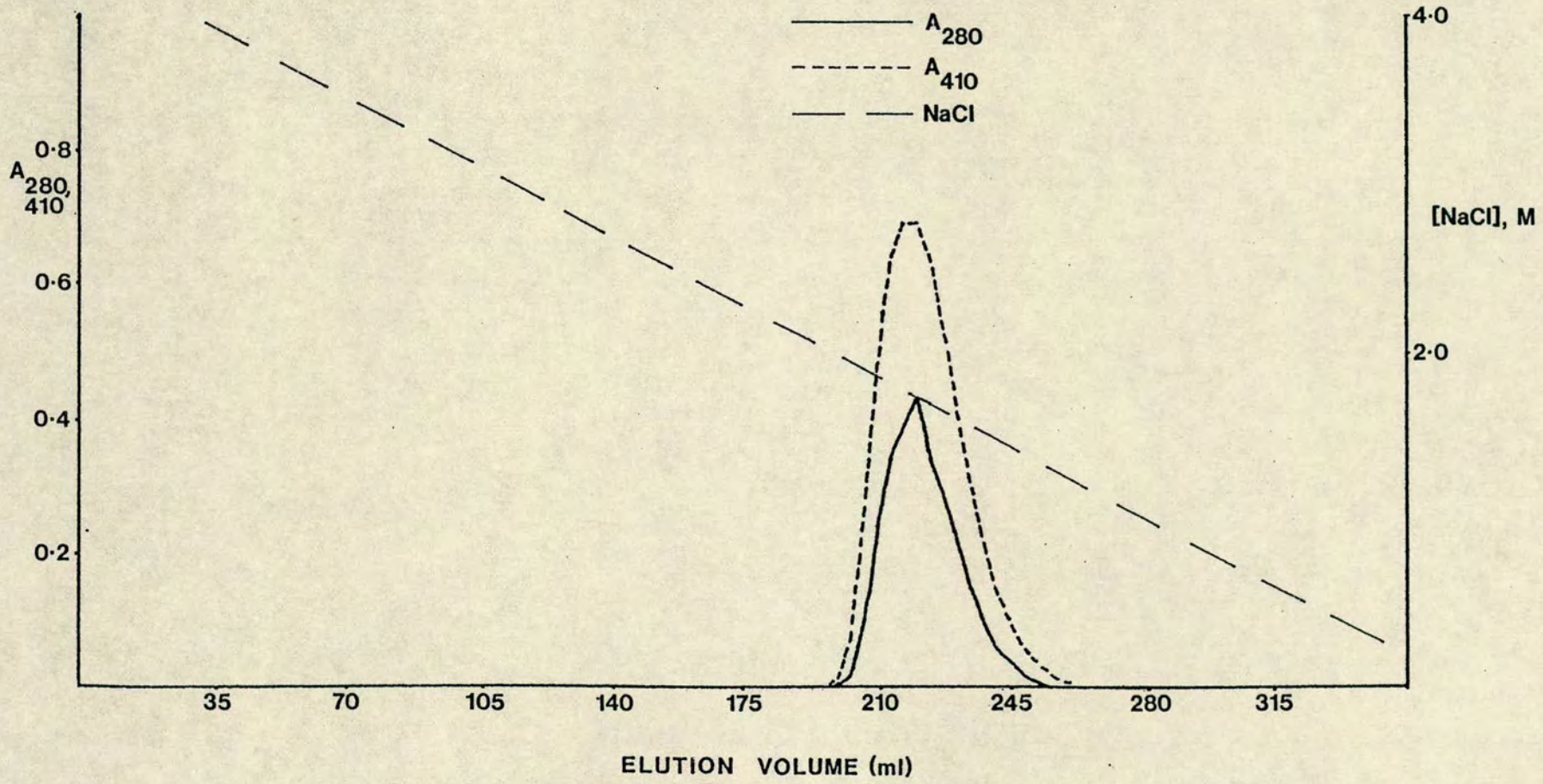


Fig. 5.2 Elution profile of hydoxyapatite flavocytochrome c fractions chromatographed on phenyl sepharose



cytochromes was determined by chromatographing a mixture of cytochromes partially purified by anion exchange chromatography (Fig. 5.3). The elution profile obtained shows that although most of the periplasmic cytochromes were eluted close to the flavocytochrome at 1.7 M NaCl, proteins were separated throughout the gradient, including a single cytochrome which was eluted isocratically after the gradient had finished. The cytochrome had a molecular weight of 20 000 d and was ascorbate-reducible.

SDS-PAGE was carried out on the pooled cytochromes following each chromatographic step to monitor the progress of purification (Plate 5.1). The purified protein was found to be electrophoretically pure (Plate 5.1) and was used for all further analyses. It had a haem content of 102 nmol ml^{-1} as estimated by the pyridine haemochrome method (Section 5.4) and a purity index (A_{552} /A_{280}) of 0.945 (Table 5.2). Table 5.1 summarises the purification procedure and gives typical yields of the flavocytochrome.

The molecular weight of the flavocytochrome was determined by SDS-PAGE with reference to the following marker proteins: horse-heart cytochrome _c_, 12 400; carbonic anhydrase, 29 000; ovalbumin, 45 000; bovine serum albumin, 67 000; phosphorylase _b_, 97 000; and -amylase, 200 000. A plot of distance migrated relative to that of a low molecular weight dye, against the log of the molecular weight for each protein, gave

Fig. 5.3 Hydrophobic interaction chromatography of a mixture of cytochromes from S. putrefaciens

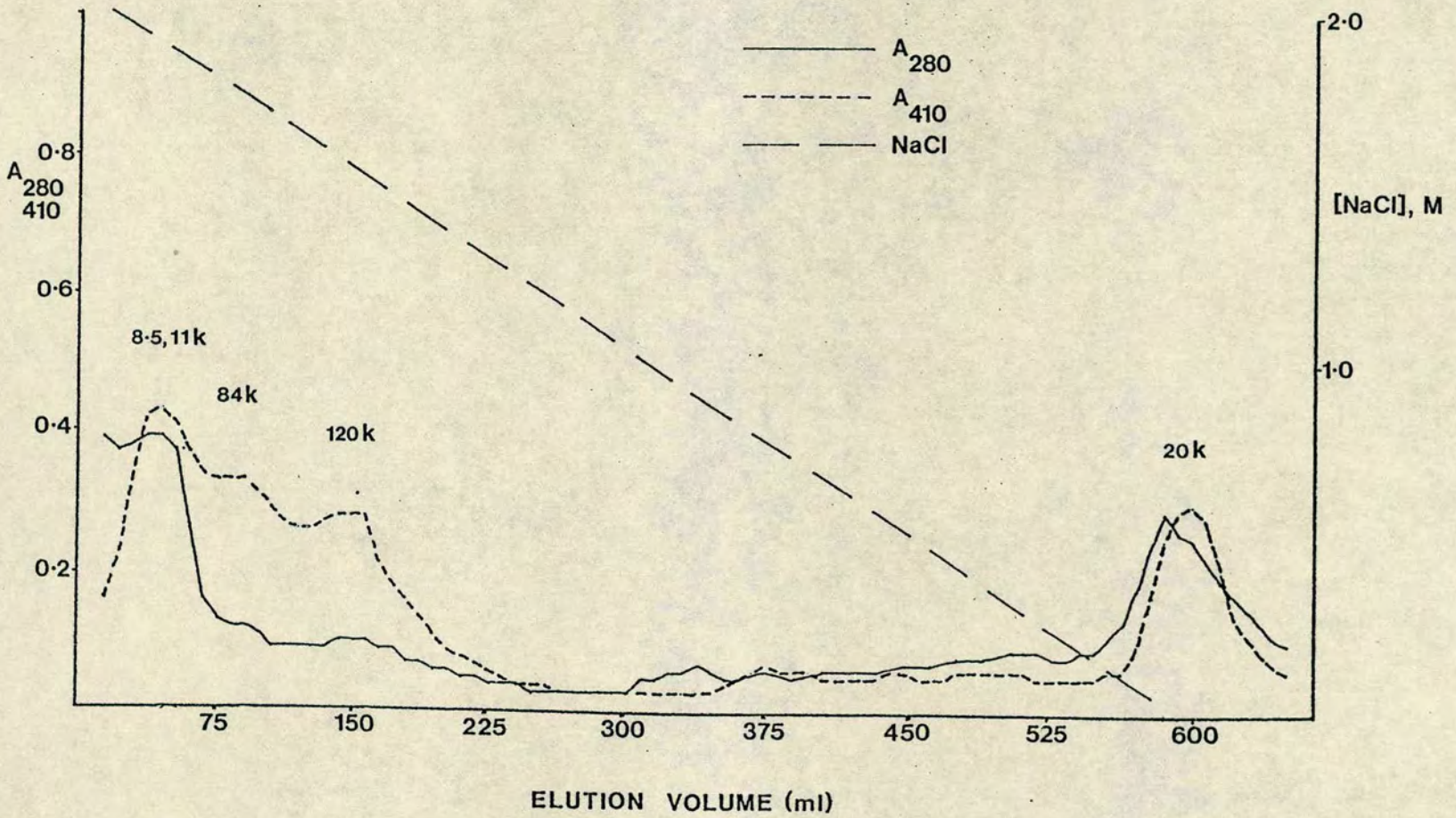
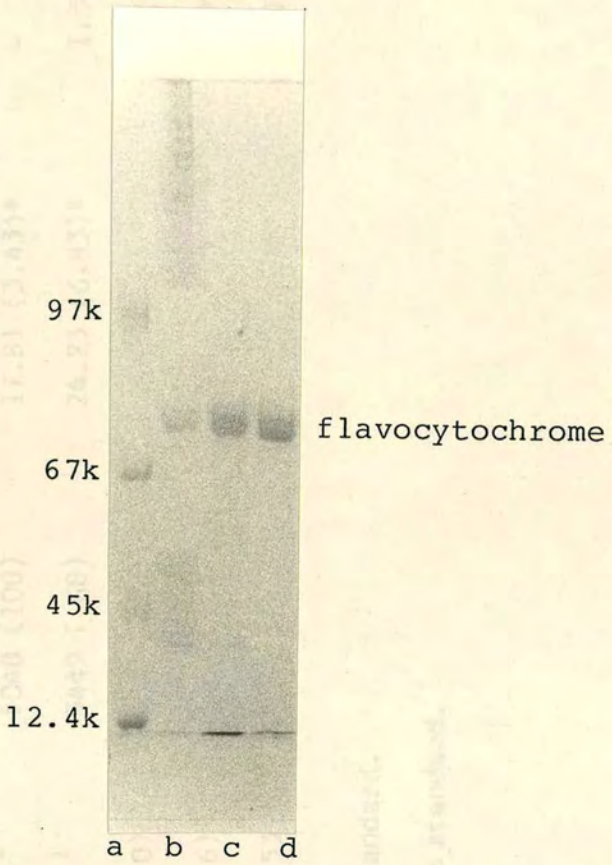


Plate 5.1 Purification of flavocytochrome c from S. putrefaciens



7.5% gel stained for protein

tracks: (a) standards
(b) ion exchange fractions
(c) hydroxyapatite fractions
(d) hydrophobic column fractions

Table 5.2. Optical properties of *S. putrefaciens* flavocytochrome *c*.

Absorption maxima (nm)			Extinction coefficients (mM^{-1} cm^{-1})	
Reduced	Oxidised	Band	Reduced	Oxidised
552	-	α	181.2	-
523	-	β	95.4	66
418	410	Soret	1129	730
-	354	δ	-	163
-	276		-	-

$\alpha_{(red)}/A_{280_{(ox)}} = 0.945$

$Soret_{(red)}/Soret_{(ox)} = 1.55$

$\alpha_{(red)}/\beta_{(red)} = 1.90$

$\alpha_{(red)}/\alpha_{(ox)} = 3.64$

Reduced minus oxidised

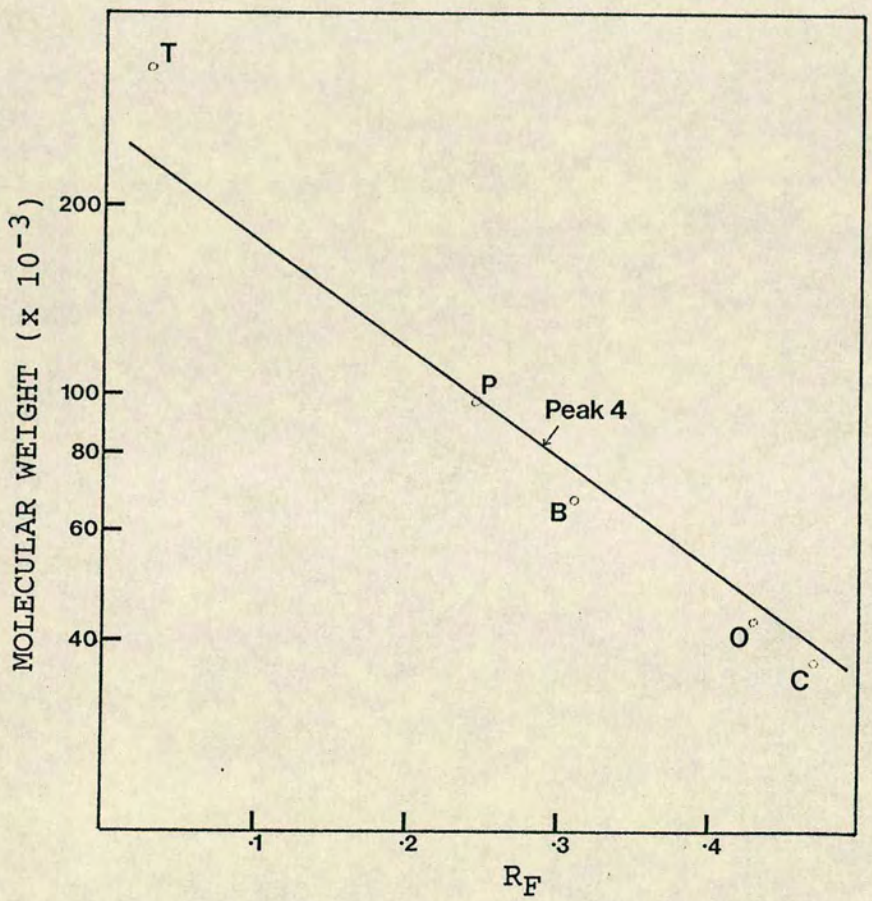
Peaks		Troughs
α	552	445
β	523	405
Soret	422	

a smooth curve (Fig. 5.4). The flavocytochrome was calculated from this curve to have a relative molecular weight of 84 000. The purified flavocytochrome corresponded well with the protein in crude extracts (Plate 5.1). No subunit structure was found when the protein was treated by boiling in the presence of reducing agents and electrophoresed in the presence of SDS, indicating that it consisted of a single polypeptide.

5.2 Optical Spectra

The purified flavocytochrome solution was diluted 100-fold for spectral studies. The cytochrome was fully oxidised as prepared: reduction of the cytochrome was achieved by additions of solid dithionite until no further spectral changes were observed. The absolute reduced and oxidised spectra of the cytochrome are shown in Fig. 5.5, and the reduced minus oxidised spectrum in Fig. 5.6. The reduced cytochrome had a sharp symmetrical α-peak at 552.3 nm, β-peak at 523 nm and Soret (ϒ) peak at 418 nm. The Soret peak had a slight shoulder on the short wavelength side at about 395 nm. The oxidised cytochrome had peaks at 410 nm, 354 nm and 276 nm. The reduced minus oxidised difference spectrum had peaks at 552.3 nm, 523 nm and 422 nm with troughs at 445 nm and 405 nm. Fig. 5.7 shows the reduced minus oxidised

Fig. 5.4 Molecular weight determination of flavocytochrome c by SDS-PAGE



T: thyroglobulin P: phosphorylase b
O: ovalbumin C: carbonic anhydrase
B: bovine serum albumin

Fig. 5.5 Absolute reduced and oxidised spectra of S. putrefaciens flavocytochrome c

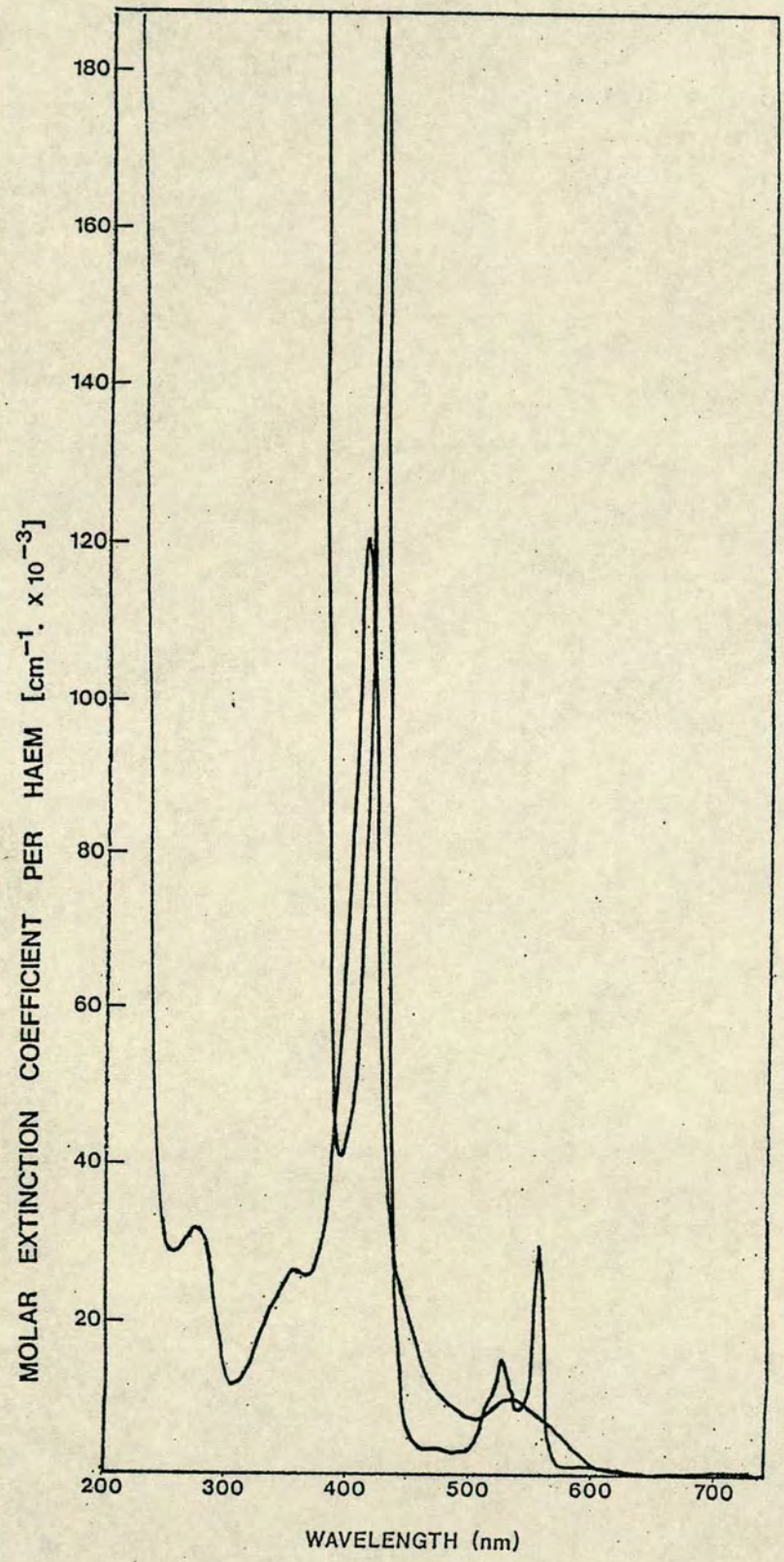


Fig. 5.6 Reduced minus oxidised difference spectrum of S. putrefaciens flavocytochrome c

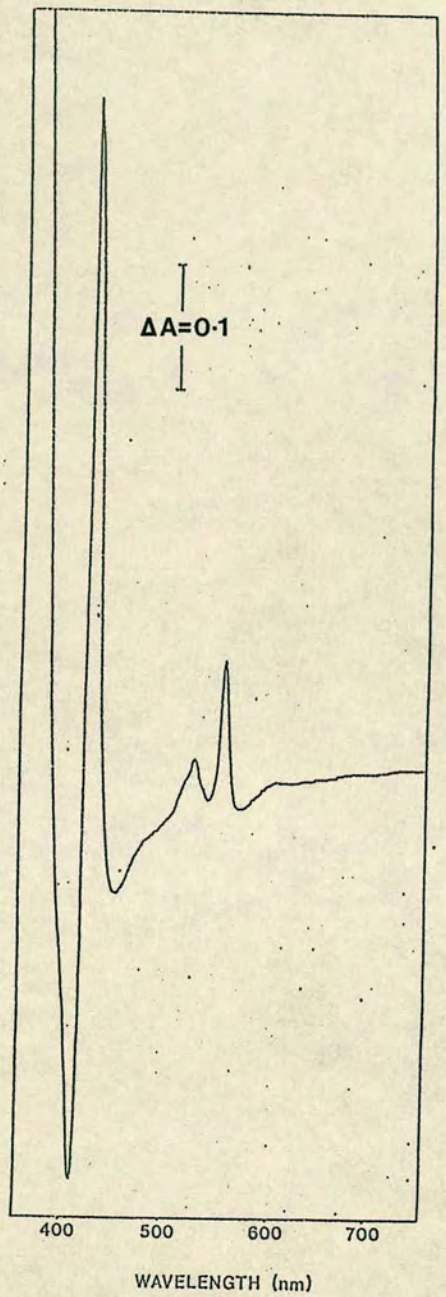
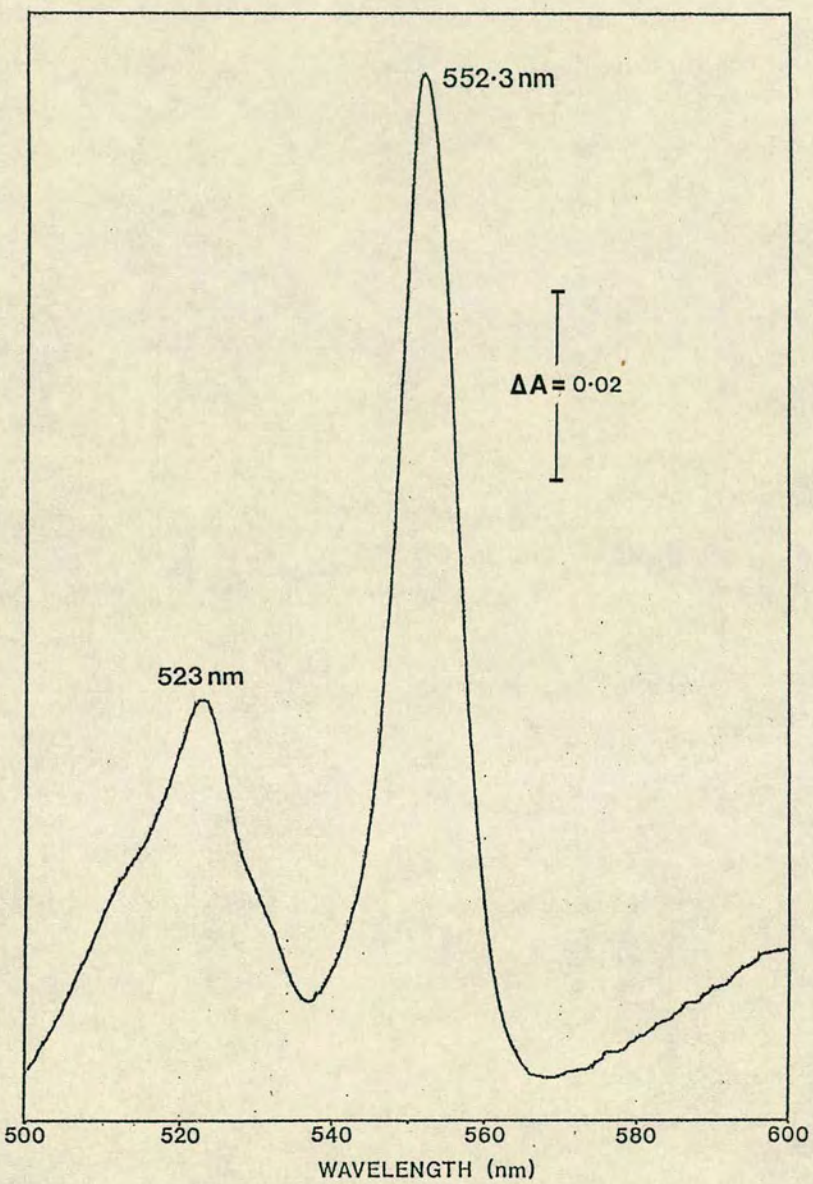


Fig. 5.7 Reduced minus oxidised difference spectrum of α- and β-peaks of S. putrefaciens flavocytochrome c



difference spectrum in the α and β region in more detail. The spectrum of the oxidised flavocytochrome from 650 nm to 750 nm with horse-heart cytochrome c as a reference is given in Fig. 5.8. No 695 band indicative of met-Fe-his coordination as in horse-heart cytochrome c, was found in the flavocytochrome.

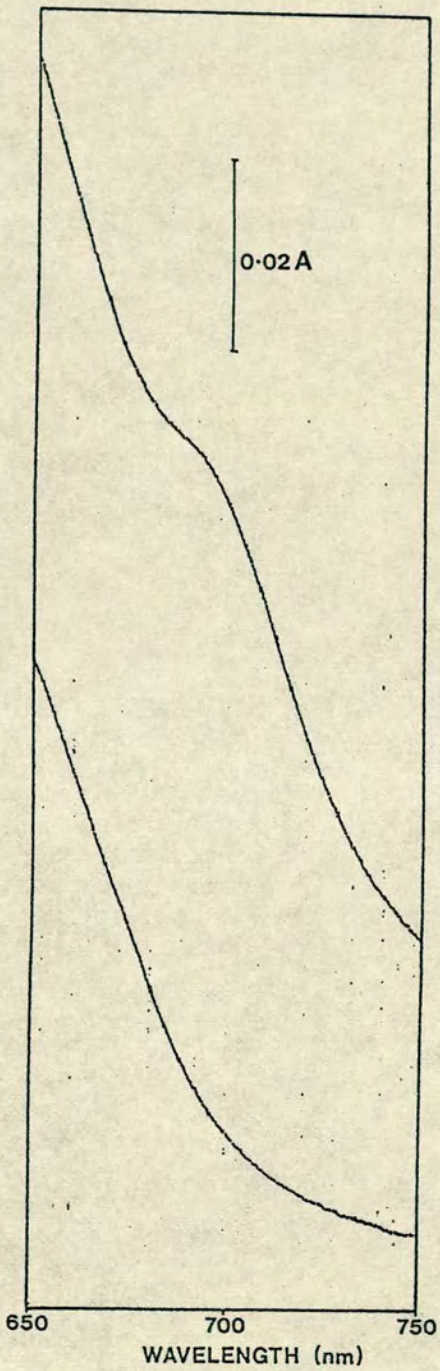
5.3 Haem Determination

Determination of haem in the flavocytochrome was carried out as in Section 2.5. The pyridine haemochrome spectrum is shown in Fig. 5.9 and is typical of the spectrum obtained for c-type cytochromes with two thioether bonds between the haem vinyls and the polypeptide chain (Section 1.4.3). There are no other peaks or shoulders in the spectrum which might indicate non-standard linkage for any of the haem moieties of the cytochrome, or presence of other haem types. The concentration of haem in the standard solution used for spectra and amino-acid analysis was 0.102 mM by this method. This figure was used for calculation of the molar absorbtivities of the spectral maxima of flavocytochrome c given in the previous section.

5.4 Flavin Determination

The flavin content of S. putrefaciens

Fig. 5.8 Determination of haem ligands in S. putrefaciens flavocytochrome c

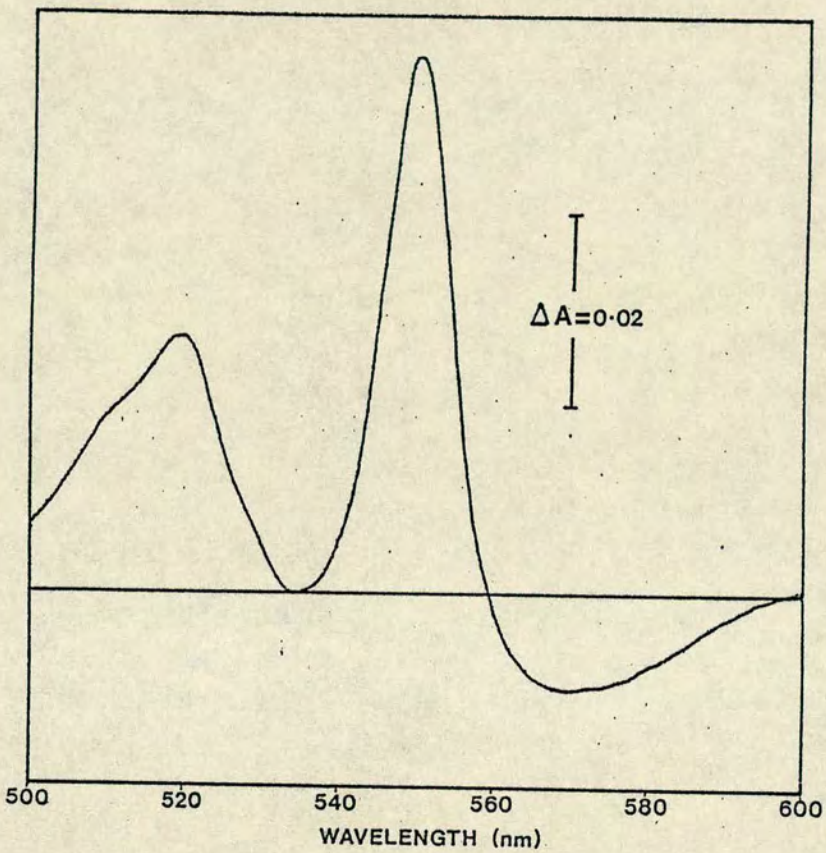


Upper trace: horse-heart cytochrome c

Lower trace: S. putrefaciens flavocytochrome c

Solutions normalised with respect to reduced α-peak height

Fig. 5.9 Pyridine derivative spectrum of S. putrefaciens flavocytochrome c



flavocytochrome c was determined spectrophotometrically as in Section 2.12. After tentatively identifying the flavin moiety on the basis of absorption maxima at 445 nm and 350 nm (see Fig. 5.10) the flavin was quantitated using the millimolar extinction coefficient for FMN at 445 nm of 12.5 cm^{-1}. The flavin concentration of solutions obtained from desalting flavocytochrome into 4.5% formic acid was determined at 1.22 µM in a volume of 10.5 ml giving 12.8 µmol FMN/700 µl flavocytochrome taken for desalting. Thus the standard solution of flavocytochrome contained 18.3 µM FMN. Using the value of 0.102 mM haem from Section 5.3 a value of 5.58 mol haem per mol FMN was obtained. The flavin was non-covalently bound since it was released from the protein under mild (denaturing) conditions (Fig. 5.11).

5.5 Amino Acid Analysis

Several samples of flavocytochrome were taken for amino acid analysis. Figures for valine and isoleucine were averaged from 70 h hydrolysis. For the labile amino acids serine and threonine, extrapolation of the 70 h and 20 h hydrolysis data to zero time was performed. Tryptophan is largely destroyed by the acid hydrolysis step, however a value of 17 residues per mol was estimated from A_{280} measurements using millimolar extinction coefficients of 1.1 cm^{-1} for tyrosine, 5.2

Fig. 5.10 Spectrum of flavin separated from S. putrefaciens flavocytochrome c

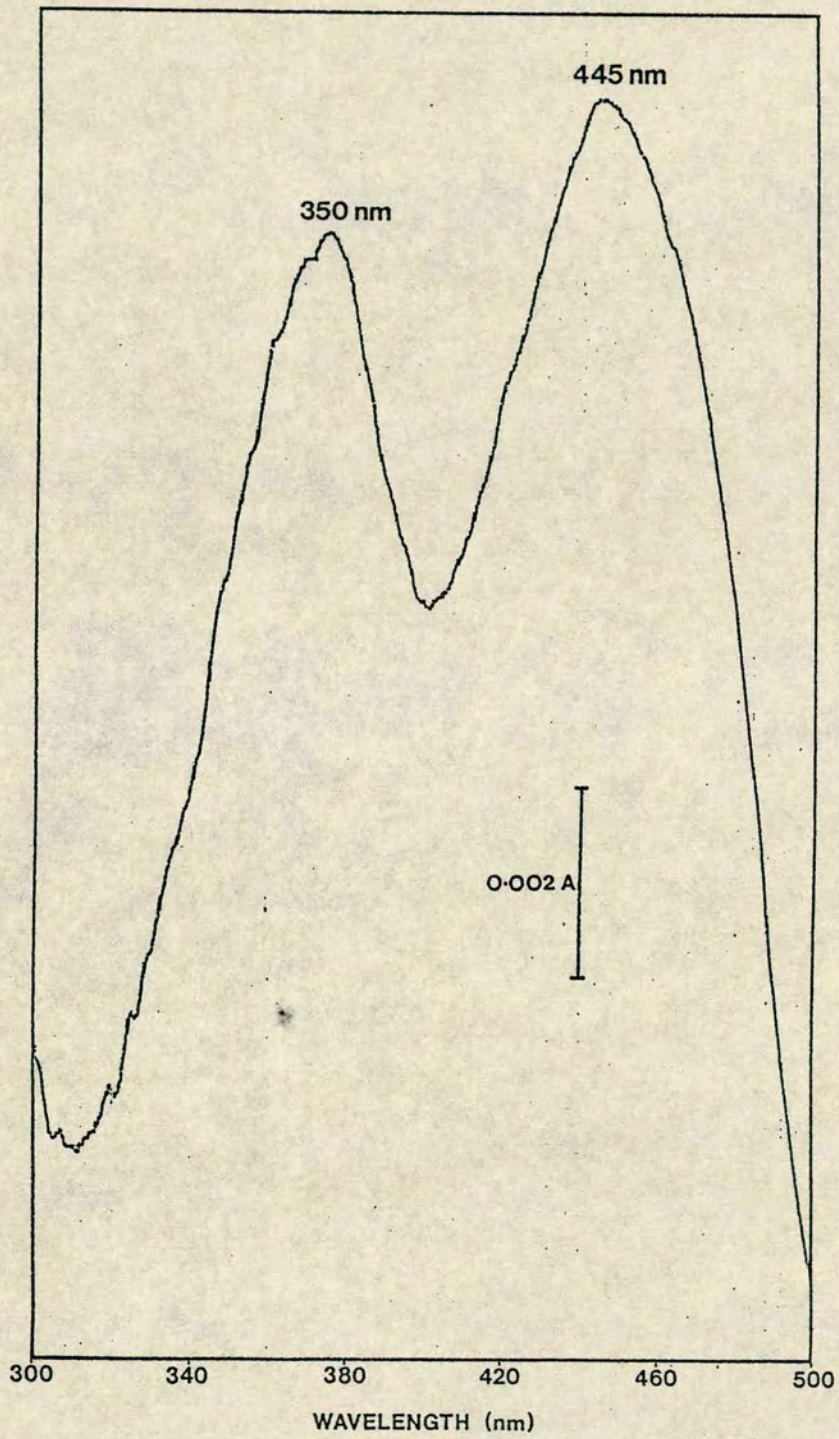
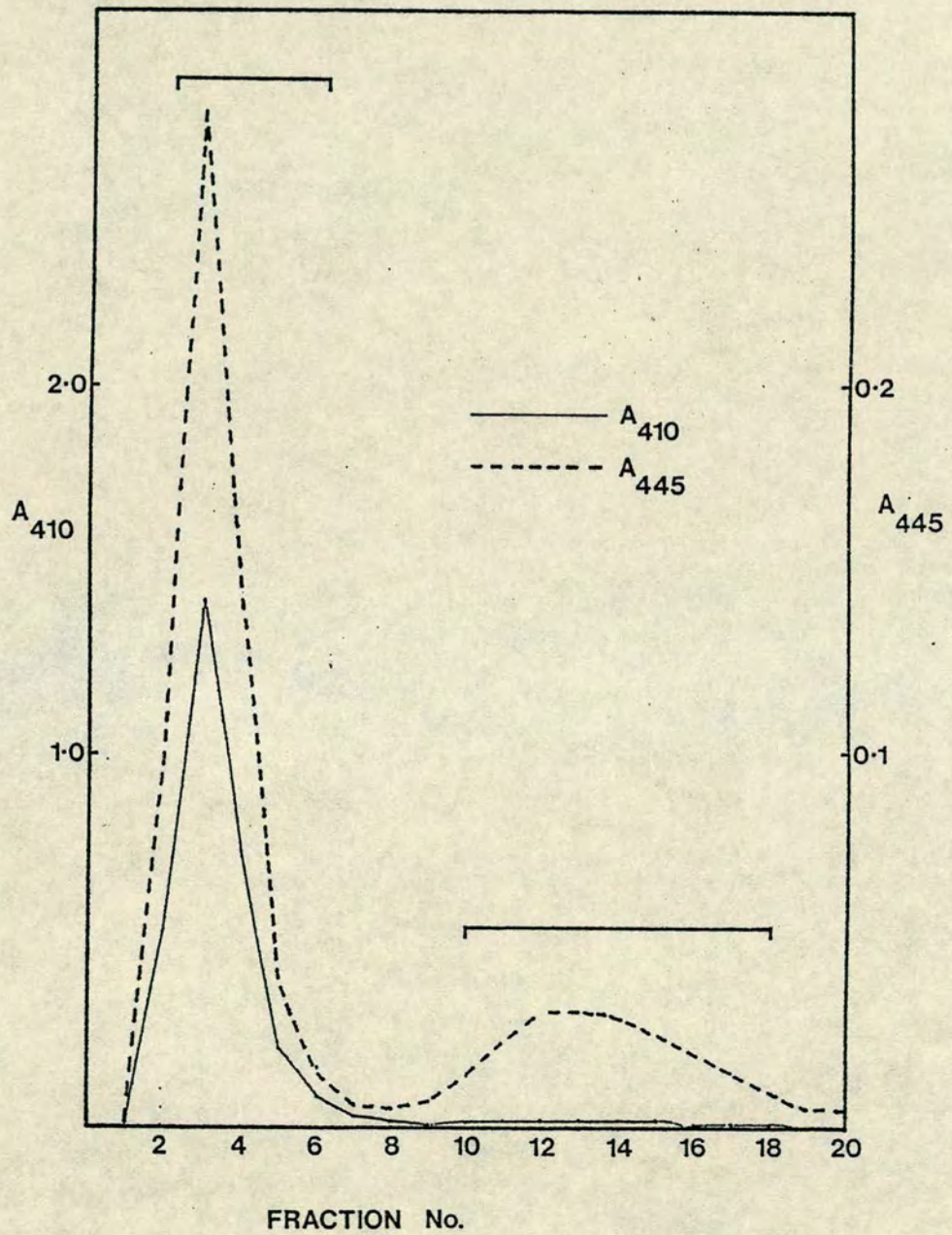


Fig. 5.11 Elution profile of flavocytochrome c chromatographed on Sephadex G25 equilibrated with 4.5% formic acid



cm^{-1} for tryptophan and 13.8 cm^{-1} for haem (ElKurdi, 1982). All other amino acid determinations were averaged from 5 runs: 20 h (2) and 70 h (2) and the control for the performic acid oxidised sample (20 h: 1).

The haem content was calculated to be 69 umol mg $protein^{-1}$ using the protein concentration from quantitative amino acid analysis and haem concentration from Section 5.3. This figure was used to calculate the haem and flavin (FMN) content of the flavocytochrome: the protein contained 5.75 mol haem and 1.03 mol flavin per mol flavocytochrome, given a relative molecular weight of 84 000 d from SDS-PAGE. Assuming an integral value of 6 for the haem content, the number of amino acid residues was calculated relative to haem content (Fig. 5.3). By amino acid analysis the molecular weight of the protein was calculated to be 89 000 d. The "purity index" A_{552}/A_{280} of 0.945 (Table 5.2) is consistent with a haem/protein ratio of 6 for a protein of 89 000 d, and further support is provided by the cysteine determination of 10-12 (Table 5.3). Hence the protein is best described as a hexahaem flavocytochrome c.

The amino acid composition was used to calculate the relative hydrophobicity and the polarity index of the protein. A hydrophobicity of 0.89 kcal.mol $protein^{-1}$ (Bigelow, 1967) and a polarity index of 0.54 (Capaldi & Vanderkooi, 1972) were obtained, indicating

Table 5.3. Amino acid analysis of *S. putrefaciens* flavocytochrome *c*.

Amino acid	Residues per mol protein*	Performic acid-oxidised		Integral residues
		Test	Control	
Asp	106.8	98.7	105.9	107
Thr	44.3	45.6	44.6	44
Ser	44.3	43.4	43.1	44
Glu	62.0	66.0	66.4	62
Pro	20.0	21.6	20.0	20
Gly	80.6	75.5	80.1	81
Ala	94.3	91.0	96.1	94
Val	66.2	60.3	61.4	66
Met	15.2	-	21.1	15
Ile	36.9	33.5	33.0	37
Leu	50.8	49.5	49.0	51
Tyr	19.7	-	20.0	20
Phe	17.0	14.8	17.4	17
His	24.3	22.9	22.7	24
Lys	55.9	58.2	56.6	56
Arg	28.7	26.0	26.3	29
Cys	-	10.2	-	10-12
Trp+	-	-	-	17
Total				722

Molecular weight of apoprotein by amino acid composition = 84 770

* See text.

\+ By absorbance at 280 nm (see text).

that the protein was extremely hydrophilic. Membrane proteins have a polarity index of ≤ 0.40 while hydrophilic proteins have a polarity index of 0.47 - 0.56 (Capaldi & Vanderkooi, 1972).

5.6 Redox Titrations

Titration of flavocytochrome with dithionite was carried out as in Section 2.9 using 10 uM final concentration of the following mediators: PMS, PES, DAD, HNQ, A2S, A26D and BV (Table 2.2). As with crude preparations (Section 3.4), the purified cytochrome showed a resistance to reduction at very low potentials ($\approx$-200 mV) making long equilibration times necessary. The redox spectrum is given in Fig. 5.12. The spectrum was typical of a c-type cytochrome and was devoid of unusual features such as shoulders or band splitting. Analysis of the titration data (Figs. 5.13 and 5.14) indicated that at least two potentially non-equivalent haems were contributing to the spectrum. The data was tentatively resolved into two components with potentials of -204 mV and -320 mV: comparison of Fig. 5.13 with Figs. 2.7 and 4.20, single component plots, confirms that at least two potentiometrically nonequivalent haems were contributing to the spectrum. It has been noted that cytochromes with multiple haems can be difficult to analyse and interpret (Pettigrew & Moore, 1986; LeGall & Forget, 1978).

Fig. 5.12 Redox spectrum of S. putrefaciens flavocytochrome c

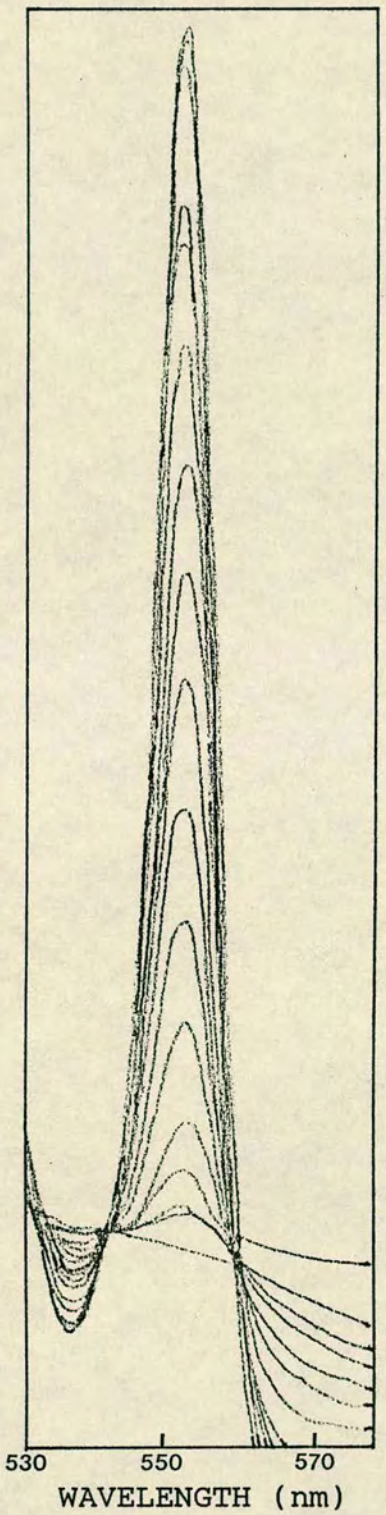


Fig. 5.13 Redox titration of S. putrefaciens flavocytochrome c

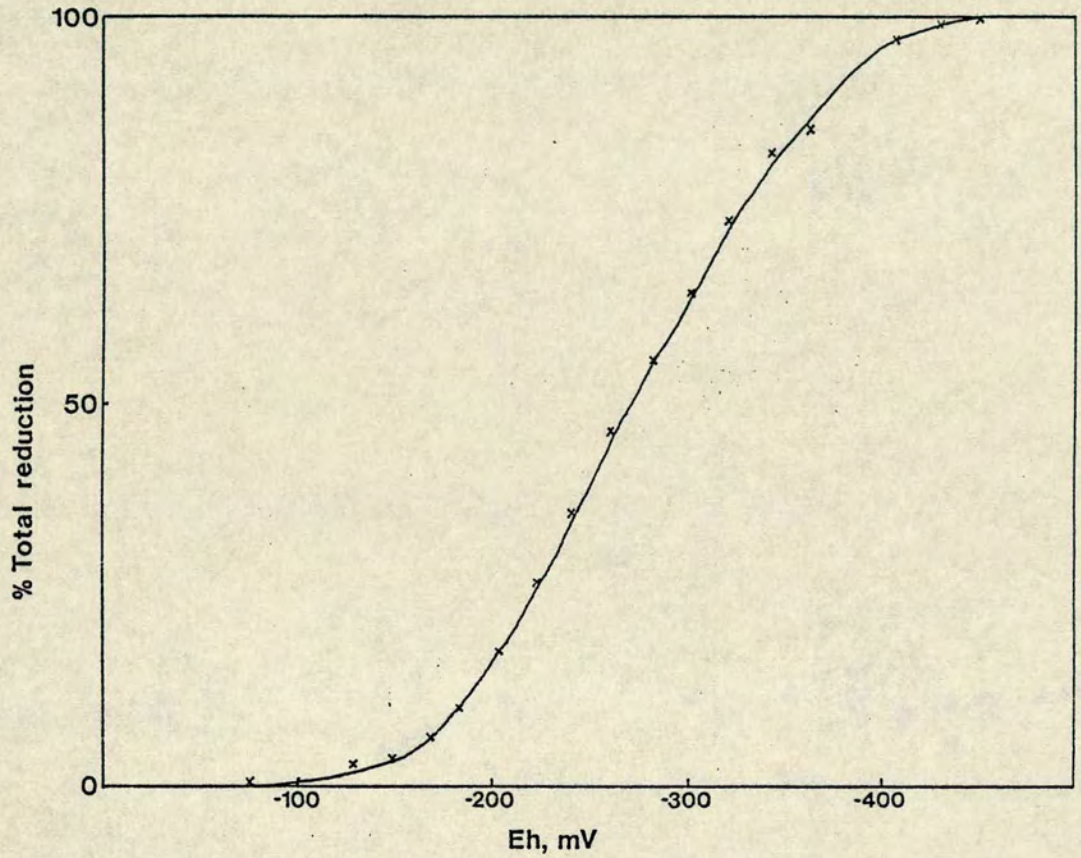
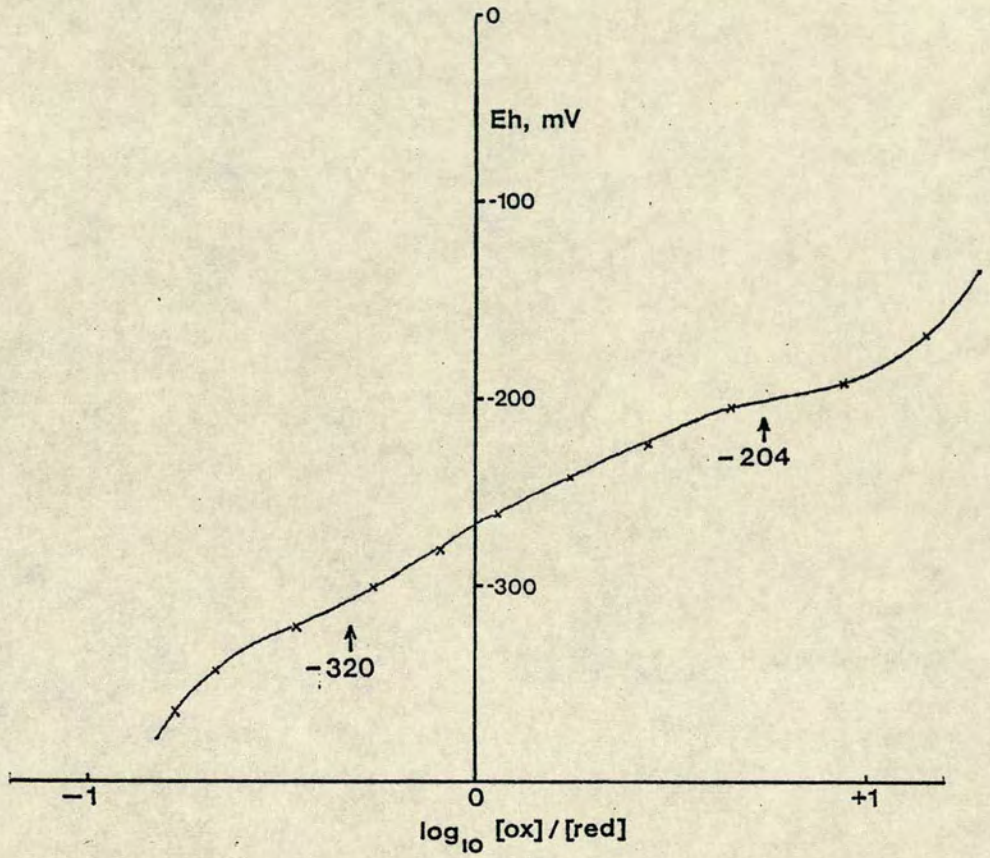


Fig. 5.14 Nernst plot of redox titration of *S. putrefaciens* flavocytochrome *c*



5.7 Enzymatic Studies

Flavocytochrome c was assayed for enzymatic activity with a number of non-proteinaceous electron acceptors using the spectrophotometric method for TMAO reductase (Section 2.4). The flavocytochrome did not react with TMAO, and no oxidation of MV^+ was observed when SO_3^{2-}, SSO_3^{2-}, NO_2^-, NO_3^- or DMSO replaced TMAO in the assay. Although the full complement of low-potential cytochromes in TMAO grown periplasm was oxidised by TMAO (Section 3.1.2), reduced preparations of flavocytochrome were not reoxidised by TMAO in the presence of TMAO reductase, indicating that at least one other component was mediating electron flow between flavocytochrome c and TMAO reductase. The flavocytochrome was not reduced by H_2, NADH or formate under anaerobic conditions, but could be reduced by formate in the presence of membrane preparations containing active formate dehydrogenase (Section 3.5).

5.8 Ligand Binding Properties

The ligands carbon monoxide and cyanide were tested for reactivity with purified cytochrome. CO was found to react rapidly with the flavocytochrome. Bubbling of preparations with CO for 30 s followed by recording the spectra at 5 or 10 min intervals showed

that binding was complete within 10 min, and that extended bubbling times did not increase the amount of CO binding. Fig. 5.15(a) shows the reduced + CO minus reduced difference spectrum. About 50% of the haem content bound CO as judged by comparison of reversed α-peak heights with the oxidised minus reduced difference spectrum (Fig. 5.15(b)) suggesting that perhaps three of the six haems were CO-reactive. Small changes were evident between the two spectra. Firstly, the CO-spectrum was devoid of the peak at 445 nm due presumably to bleaching of the flavin by dithionite. Secondly, the peak at 405 nm in the oxidised minus reduced difference spectrum was shifted 7 nm to the red. Thirdly, a small shoulder was evident at 395 nm. Numerical details are given in Table 5.4.

Cyanide was found to be titratable with the cytochrome. Initially, the addition of aliquots of cyanide to an oxidised preparation of flavocytochrome against an oxidised reference caused rapid and stable changes in the spectrum (Fig. 5.16). Spectral changes were complete within a few seconds of each pulse, and further changes were not observed with time. Above 50 nM total CN^-, the spectrum continued to change slowly for about 1 h. After this period, further additions of CN^- caused no spectral changes. In the fully developed spectrum (Fig. 5.16(a)) major peaks were apparent at 295 nm and 421 nm with minor peaks at 450 nm and 545 nm. The major trough was at 405 nm with

Fig. 5.15 CO difference spectrum and oxidised minus reduced difference spectrum of flavocytochrome c

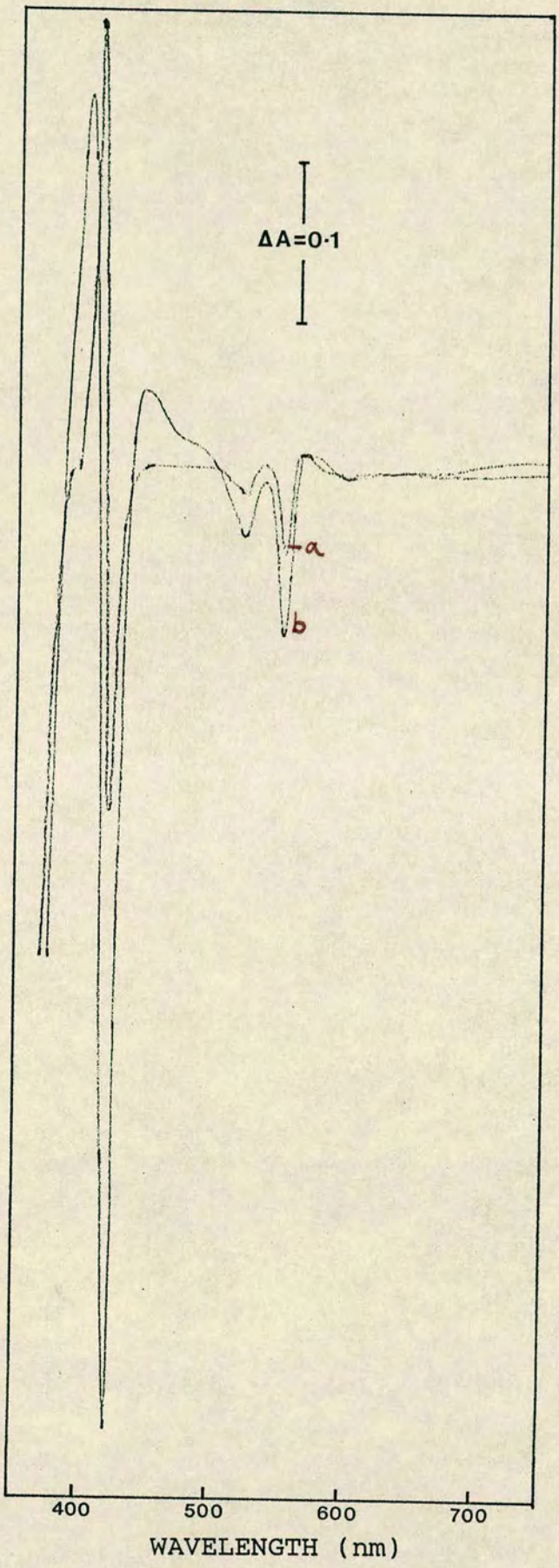


Fig. 5.16 CN^- difference spectrum of S. putrefaciens flavocytochrome c: CN^- titration

Final CN^- concentration = 50 mM

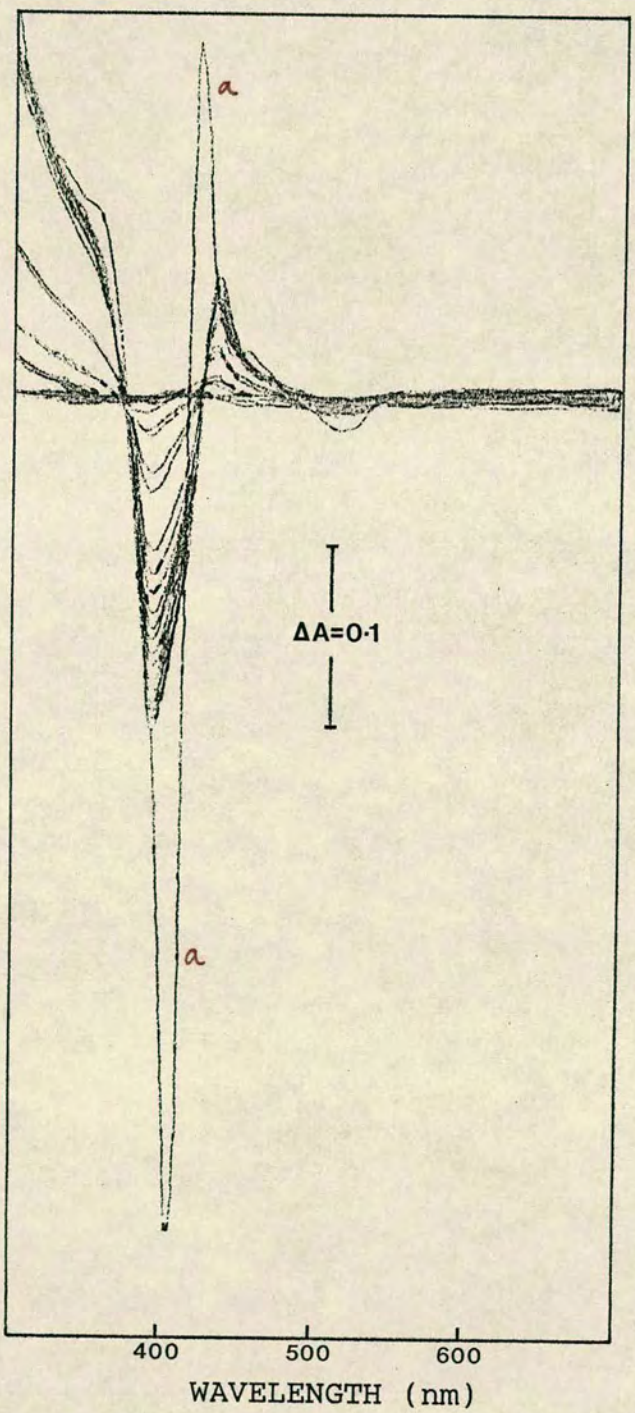


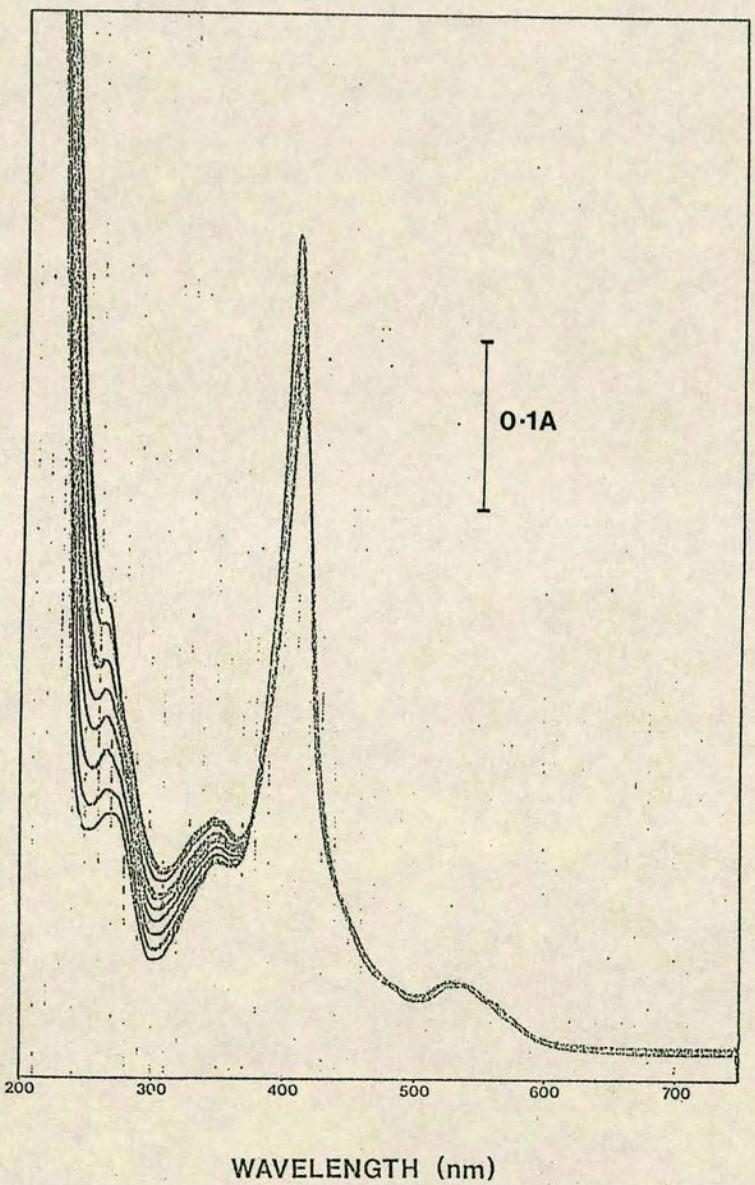
Table 5.4. Optical properties of carbonomoxy-flavocytochrome c from S. putrefaciens

Reduced + CO minus reduced (nm)		Oxidised minus reduced (nm)	
Peaks	Troughs	Peaks	Troughs
412	552	445	552
	523	405	523
	422		422

Table 5.5. Optical properties of CN^--flavocytochrome c

Oxidised + CN^- minus oxidised		Oxidised + CN^-	
Peaks	Troughs	Peaks	Troughs
546	562	525	300
450	516	415	500
421	405	354	
295			

Fig. 5.17 CN^- titration spectrum of S. putrefaciens flavocytochrome c



minor troughs at 515 and 560 nm. The major spectral features at 421 nm and 405 nm were similar to those in reduced minus oxidised difference spectra at 422 and 405 nm, respectively.

An unusual feature of the CN^- titration was the occurrence of spectral shift. The major peak first appeared at about 430 nm, shifted to the red (433 nm) during the early course of the titration, then shifted back towards the blue to 421 nm. The 405 nm trough appeared first at 375 nm and shifted progressively to the red during the titration.

Formation of a broad charge transfer band in the near infrared at about 670 nm, typical of other flavocytochromes c (Meyer & Kamen, 1982) was not observed. Numerical details for the spectra are given in Table 5.5. Fig. 5.17 shows a titration spectrum of the flavocytochrome against a buffer blank.

5.9 Discussion

5.9.1 Optical Properties

The spectrum of Shewanella putrefaciens flavocytochrome c is typical of the Class III cytochromes found in photosynthetic and sulphate reducing bacteria (Meyer et al., 1971) and showed:

a) a shoulder on the short wavelength side of the reduced Soret band

b) a high Soret (red.)/Soret (ox.) ratio (1.55)

c) no 695 nm band in the oxidised form.

In common with other Class III cytochromes, the flavocytochrome contained multiple haems of very low redox potential. Table 5.6 gives a comparison between the spectral properties of the flavocytochrome (Fig. 5.5), a typical cytochrome c_3 from Desulfovibrio gigas (Fig. 5.18), and the c_{552} hexahaem nitrite/hydroxylamine reductase of E. coli K12 (Fig. 5.19). The extinction coefficient of the flavocytochrome and the c_3 from D. gigas are extremely similar, while those of the nitrite reductase are rather different, in particular the reduced Soret band which is appreciably lower. The absorption bands of the three cytochromes are, however, quite similar. It is noteworthy that the greatest difference observed between the flavocytochrome and the c_3, at 354 nm, covers a region of the spectrum at which flavin absorbs. The spectrum of S. putrefaciens flavocytochrome c is quite different from the flavocytochrome c found in some phototrophic bacteria and in Pseudomonas putida (Table 5.7 and Fig. 5.20). These have prominent shoulders on the oxidised Soret band at 450 nm and 475 nm due to the flavin moiety, which bleach on reduction. They also have a prominent 280 nm protein band due to the low haem/protein ratio. The S. putrefaciens flavocytochrome in contrast has a very small 280 nm

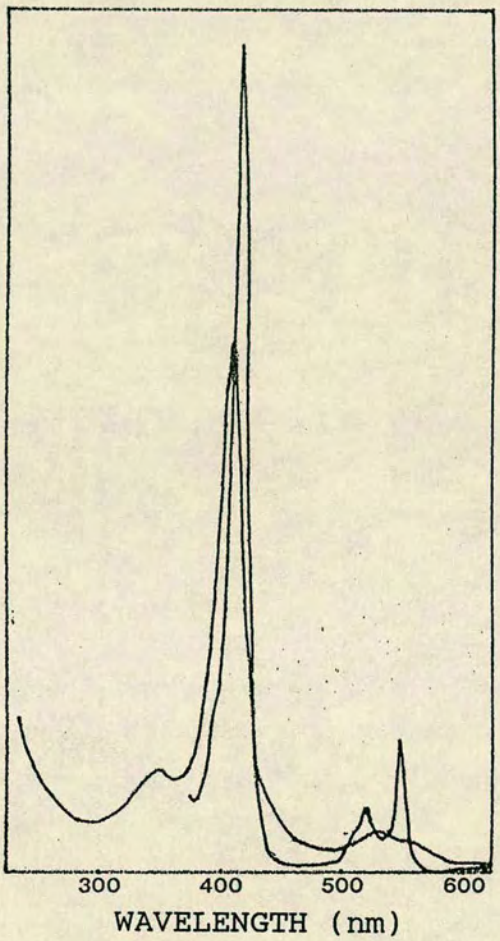
Table 5.6. Extinction coefficients (M^{-1} cm^{-1} x 10^3) per mole of haem of *S. putrefaciens* flavocytochrome *c*, *Desulfovibrio gigas* c_3 (Mr 26 000) and *E. coli* c_{552} hexahaem nitrite reductase.

Flavocytochrome *c*		[1] c_3 (26 000)		[2] c_{552} nitrite reductase	
Oxidised	Reduced	Oxidised	Reduced	Oxidised	Reduced
-	30.2 (552)	-	31.6 (552)	-	28.8 (551)
11.0 (530)	15.9 (523)	11.1 (530)	16.1 (526)	10.5 (531)	17 (523)
121.6 (410)	188.2 (418)	121 (410)	187 (420)	97 (408)	122.3 (419)
27.1 (354)	-	23.4 (352)	-	28.5 (355)	-

[1] LeGall & Forget, 1978

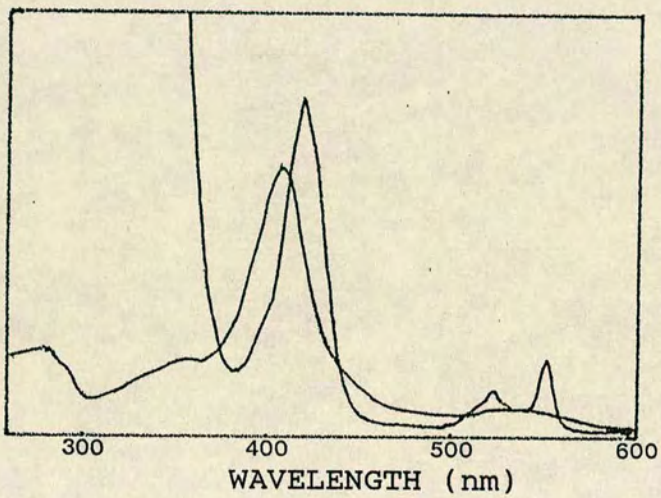
[2] Kajie & Anraku, 1986

Fig. 5.18 Absolute reduced and oxidised spectra of *Desulfovibrio gigas* cytochrome c_3



Redrawn from Mayer & Kamen, 1982.

Fig. 5.19 Absolute reduced and oxidised spectra of *E. coli* hexahaem cytochrome c_{552} nitrite reductase



Redrawn from Kajie & Anraku, 1986

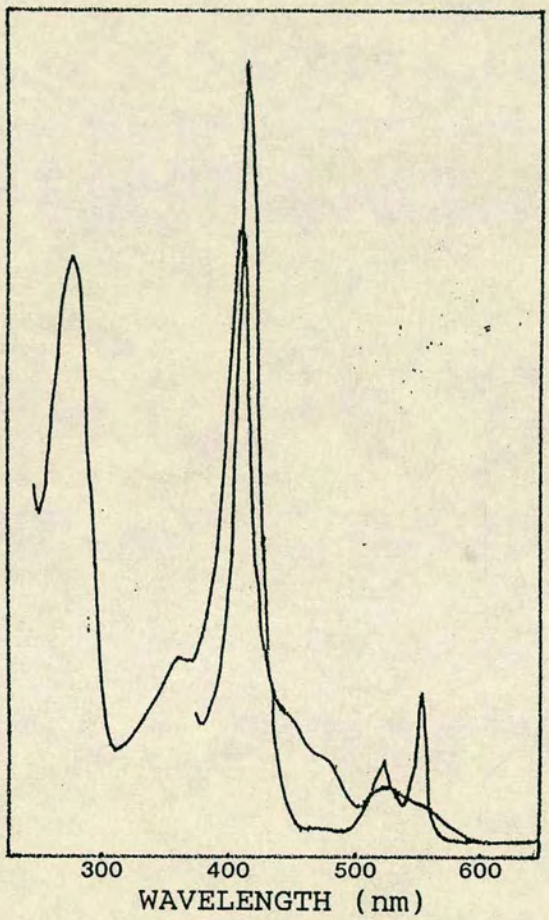
Table 5.7. Properties of flavocytochrome c.

Source	Mr x 10^{-3}	Subunit structure and prosthetic groups	Redox potential	Flavin binding
Chromatium spp.*	72	20-21K dihaem 45-46K 1 FAD	80,29 mV	8-α-cysteinyl-8-α-hydroxyl-flavin
Chlorobium spp.*	50 59	11K haem 47K flavin	98 mV	"
Ps. putida++	115	56K monohaem 56K monoflavin		o-tyrosyl-8-α-flavin
S. putrefaciens.	84	84K 6 haems, 1 flavin	-200, -300 mV	noncovalent

*After Wood, 1984

++ After Meyer & Kamen, 1984

Fig. 5.20 Absolute reduced and oxidised spectra of flavocytochrome c from phototrophic bacteria



Redrawn from Meyer & Kamen, 1982

peak and slight shoulders at about 450 nm and 480 nm, attributed to the high relative haem content partially masking the flavin and protein bands.

5.9.2 Structural Properties

The flavocytochrome c of S. putrefaciens was shown to consist of a single polypeptide chain with a molecular weight by amino acid analysis of 89 000. Few c-type cytochromes have been discovered of this chain size. Desulfovibrio vulgaris contains a c_3 of 70 000 or 85 000 Mr of unknown function (Meyer & Kamen, 1982), and a number of nitrite reductases have been shown to be high molecular weight proteins containing haem c as prosthetic group. E. coli K12 grown anaerobically on nitrate synthesises a c_{552} hexahaem protein with a relative molecular weight of 69 000 d (Kajie & Anraku, 1986) which functions as a nitrite/hydroxylamine reductase. D.desulfuricans contains a 66 000 d c-type nitrite reductase (Liu & Peck, 1981) and Achromobacter fischeri contains a c-type nitrite reductase of 80 000 d consisting of two subunits having one haem each (Meyer & Kamen, 1982).

Six mesohaems are covalently bound to the polypeptide chain of the S. putrefaciens flavocytochrome. The extraplanar 5th and 6th coordination positions of the haem ions are provided by histidyl residues, in contrast with the more usual

his-Fe-met found in most (Class I) cytochromes. Multiple-haem containing cytochromes are not uncommon in bacteria where they have been found as electron donors to multivalent reactions involving inorganic compounds. A good example of this is the 6-electron reduction of nitrite to ammonia by the nitrite/hydroxylamine reductase of E. coli described above.

The environments of the 6 haems of the S. putrefaciens flavocytochrome appear to be quite different. Half of the haem content binds CO, a feature which is well-established amongst those cytochromes which have been isolated from the periplasmic or soluble fractions of Gram-negative bacteria (Bartsch, 1967; Jones, 1972; Yagi & Maruyama, 1971; Appleby, 1969; Iwasaki & Shidara, 1969; Barrett & Sinclair, 1967; Weston & Knowles, 1977; Wood, 1984). This observation is similar to that found with the Chromatium flavocytochrome c where half of the haem content binds CO. It is not known whether one of the haems binds all of the CO, or if the CO is shared between them.

The multiple redox potentials observed with the S. putrefaciens flavocytochrome support the possibility of distinct haem environments. Transition from full oxidation to full reduction occurred over a span of 120 mV, twice that observed for a single-electron redox component. It is interesting to speculate that the

haems which bind CO might have a different potential to those which do not. Since both CO-binding capacity and the midpoint potential of the haem group(s) of *c*-type cytochromes are a function of the protein environment surrounding the haem (Pettigrew & Moore, 1987) this would not seem unlikely. Further information may be obtained by performing redox titrations in the presence of CO (Reid & Ingledew, 1979).

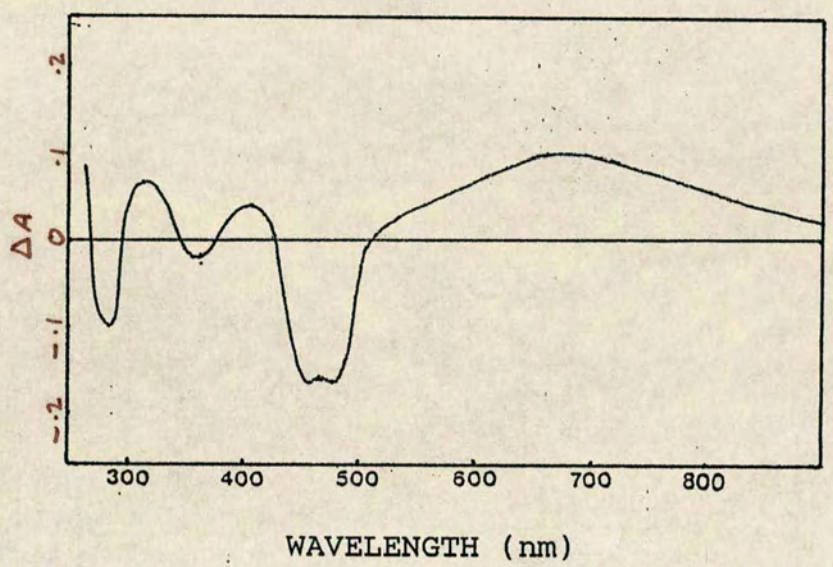
Multiple haems with different potentials are not unknown. The trihaem cytochrome c_3 (Meyer *et al.*, 1971) has one haem with a potential of -150 mV and two of -230 mV (Fiechtner & Kassner,1979). The redox potential of the flavocytochrome *c* of *S. putrefaciens* is very different from that of the phototrophic bacteria. The *Chromatium* flavocytochromes *c* have potentials of about 8-28 mV, while those of the *Chlorobium* flavocytochromes *c* are somewhat higher at 98 mV. In each case, the haems and the flavin display similar potentials. The flavin from the *S. putrefaciens* flavocytochrome is difficult to titrate spectrophotometrically because the flavin bands are largely masked by the high relative absorption of the multiple haems. An alternative method, perhaps spectrofluorimetric redox titrimetry, might yield the potential of the flavin group, which is important in context of the proposed function of the flavocytochrome (Section 6.4).

The phototrophic bacterial flavocytochromes *c*

react with nucleophilic reagents to form flavin adducts which in turn form charge-transfer complexes with the protein (Meyer & Bartsch, 1976; Meyer & Kamen, 1982). Sulphite, thiosulphate, CN^- and mercaptans bleach the flavin absorbance at 360, 450 and 480 nm and give rise to new bands in the UV at 305 nm and in the near IR at 650-700 nm (Fig. 5.21). Binding of CN^- to the phototrophic bacterial flavocytochrome c does not produce changes in the optical spectrum in the haem region. The S. putrefaciens flavocytochrome c was assayed for reactivity with a number of inorganic compounds under conditions which should have revealed any reductase activity with sulphur compounds, including the nucleophiles above, and with nitrite and nitrate. CN^- binding was observed spectrophotometrically. No reductase (dehydrogenase) activity was observed despite the similarities between the flavocytochrome and the c_{552} nitrite/hydroxylamine reductase of E. coli, and the c_3 cytochrome of D. gigas which are reactive with oxidised sulphur compounds. Cyanide bound to the flavocytochrome but in contrast with the flavocytochrome of the phototrophic bacteria, the spectral changes were attributed to haem binding rather than flavin adduct formation. The structure and properties of the bacterial flavocytochromes are given in Table 5.7.

The amino acid composition of the flavocytochrome is compared with that of several other bacterial c-type

Fig. 5.21 Absorption spectrum of charge transfer complex of phototrophic bacterial flavocytochrome c



Redrawn from Mayer & Kamen, 1982.

cytochromes in Table 5.8. Some similarities can be observed with the two nitrite reductases, which are very similar to each other, but not between the flavocytochrome and either of the c_3 cytochromes of *D. gigas*. This is emphasised in Table 5.9 where the amino acid compositions have been converted to relative contributions: ideally the more conclusive method of pairwise analysis would have been performed.

5.9.3 Location of S.putrefaciens Flavocytochrome c

The flavocytochrome was found in all cell types of *S. putrefaciens* examined by SDS-PAGE with cytochrome staining, although the amount found in aerobic cells was very small compared with the other aerobic cytochromes (Section 3.2). The flavocytochrome was found principally in the periplasmic fraction with a small amount associated with the membrane fractions. Preparation of membrane fractions in the presence of EDTA ("EDTA-extracted membranes) significantly decreased the amount of flavocytochrome present (results not shown). This would suggest that the flavocytochrome is (a) induced under oxygen-limiting conditions and during growth on nitrate, fumarate and TMAO, and (b) is loosely attached to the outer aspect of the cytoplasmic membrane. The location of c_{552} in *S. putrefaciens* as shown in Table 5.10 was deduced by Easter (1982) and is supported by the very low

Table 5.8. Amino acid composition of some bacterial cytochromes c.

Amino acid	S. putrefaciens.	Desulfovibrio gigas		Azotobacter vinelandii			Thiobacillus thiooxidans	Campylobacter sputorum	E. coli	D. desulfuricans
	Flavocytochrome c	c_3 13K[1]	c_3 26K[1]	c_4[2]	c_{551}[2]	c_{555}[2]	c-protein[3]	c_{553}[4]	c_{552} 69K nitrite reductase	66K nitrite reductase
Lys	56	17	19	11	8	8	14	9	50	47
His	24	8	16	4	2	1	7	1	20	19
Arg	29	0	11	6	1	2	16	0	24	26
Asp	107	18	21	26	8	15	23	12	60	74
Thr	44	5	5	10	8	3	12	4	35	26
Ser	64	6	15	11	7	5	14	2	24	18
Glu	62	4	23	21	13	4	41	3	83	70
Pro	20	4	11	13	5	3	27	5	28	35
Gly	81	11	20	28	10	15	37	6	44	41
Ala	94	9	17	34	12	18	34	11	75	61
Cys	12	8	16	4	2	2-4	4	2	12	12
Val	66	6	11	6	5	4	30	4	35	34
Met	15	0	5	6	1	2	6	4	27	25
Ile	37	4	8	6	4	1	15	4	29	17
Leu	51	4	4	15	6	10	38	4	41	44
Tyr	20	2	4	6	3	1	8	4	13	25
Phe	17	1	8	5	1	0	22	1	19	20
Try	17	1	ND	0	0	1	ND	0	9	5
Total	772	108	205	212	99	97	338	76	628	599

ND = Not determined.

[1]LeGall & Forget (1978).
[2]Campbell et al. (1973).
[3]Yoshida et al. (1984).
[4]ElKurdi (1982).
[5]Kajie & Anraku (1986).
[6]Liu & Peck (1981).

Table 5.9. Relative contributions of amino acids in some bacterial cytochromes c.

Amino acid	S. putrefaciens flavocytochrome c	Desulfovibrio gigas c_3 13 K	Desulfovibrio gigas c_3 26K	Nitrite reductases E. coli c_{552}	Nitrite reductases D. desulfuricans
Lys	7.25	15.74	9.27	7.96	7.85
His	3.11	7.40	7.80	3.18	3.17
Arg	3.76	0	5.37	3.82	4.34
Asp	13.86	16.67	10.24	18.29	12.35
Thr	5.70	4.63	2.44	5.57	4.34
Ser	5.70	5.56	7.32	3.82	3.01
Glu	8.03	3.70	11.22	13.22	11.69
Pro	2.59	3.70	5.37	4.46	5.84
Gly	10.49	10.19	9.76	7.01	6.84
Ala	12.18	8.33	8.29	11.94	10.18
Cys	1.55	7.41	7.80	1.91	2.00
Val	8.55	5.56	5.37	5.57	5.68
Met	1.94	0	2.44	4.30	4.17
Ile	4.79	3.70	3.90	4.62	2.84
Leu	6.61	3.70	1.95	6.53	7.35
Tyr	2.59	1.85	1.95	2.07	4.17
Phe	2.20	0.93	3.90	3.03	3.34
Trp	2.20	0.93	ND	1.43	0.83

Table 5.10. Cellular location of selected proteins in S. putrefaciens.

Cell fraction	Alkaline phosphatase			Succinate dehydrogenase			TMAO reductase			Cytochrome c_{552}		
	Total activity	%	Specific* activity	Total activity	%	Specific* activity	Total activity	%	Specific* activity	Total (nmol)	%	nmol mg^{-1} protein
Sonicate	8.71	100	173	4.27	100	33	411	100	3.18	315	100	2.44
Periplasm	5.91	68	103	0.00	0	0	295	72	5.15	253	80	4.41
Sphaeroplast	2.80	32	15	4.55	106	249	143	35	0.78	81	25	0.44
Membrane	0.17	2	18	3.42	80	352	9	2	0.92	46	15	4.79
Cytoplasm	1.92	22	51	0.00	0	0	78	19	2.07	46	15	1.20

*Specific activity = nmol min^{-1} mg $protein^{-1}$.

hydrophobicity of the protein. This observation is in agreement with Wood (1983) who has suggested that all bacterial c-type cytochromes are periplasmic proteins.

A discussion of the proposed function of the flavocytochrome is reserved for the next chapter.

CHAPTER 6.

DISCUSSION

6.1 The physicochemical properties of c-type cytochromes of S. putrefaciens

The properties of the cytochromes resolved by this study are summarised in Table 6.1, which includes some of the properties of cytochromes $\underline{c}_{551}$, $\underline{c}_4$ and $\underline{c}_5$ for comparison. Peak 3, the high-potential cytochrome found in both aerobic and anaerobic cells, is similar to cytochrome $\underline{c}_{551}$ in terms of molecular weight, redox potential and spectral maxima, although the α/β ratio (1.20) is somewhat lower than that for cytochrome $\underline{c}_{551}$ (1.7-2.1).

The 20K cytochrome shares several characteristics with cytochrome $\underline{c}_4$ including molecular weight, α/β ratio and redox potential, and in common with $\underline{c}_4$ from Azotobacter vinelandii it appears to the primarily associated with the membrane, from which it can be easily displaced by treatments which disrupt the membrane. Suggested further work on this cytochrome would entail determination of the number of haems and the redox potential, and examination of the Soret band in the oxidised and reduced state to characterise the unusually high $\text{Soret}_{red}/\text{Soret}_{ox}$ ratio.

Peak 3(a), a minor but variable component, appears to be similar to cytochrome $\underline{c}_5$ in terms of molecular weight, spectral properties and high redox potential. As discussed in Section 1.2.5, the trio of cytochromes $\underline{c}_{551}$, $\underline{c}_4$ and $\underline{c}_5$ are found in a number of bacteria

multiple low-potential cytochromes in bacteria. This is probably a reflection of a necessity in most organisms for membrane-bound components at the reducing end of the respiratory chain operating between a membrane-bound primary dehydrogenase and a membrane-bound quinone, where both the dehydrogenase and the quinone are active in proton translocation.

6.2 Characterisation of S. putrefaciens Flavocytochrome c and Comparison with Other Bacterial Species

Flavocytochrome c of S. putrefaciens was isolated as an 89 000 d hexahaem low-potential Class III-type cytochrome with a single noncovalently bound flavin group (probably FMN). The presence of a flavin group in a c-type cytochrome is unusual: they have been found in a few species of phototrophic bacteria and in Pseudomonas putida. Certain Chlorobium spp. contain a flavocytochrome c (Cusanovich & Meyer, 1982) which is active as a sulphide: cytochrome c oxidoreductase (possibly also as a sulphite and thiosulphate oxidase). These 60 000 d cytochromes consist of an 11 000 d monohaem unit with a redox potential of +98 mV and a 47 000 d monoflavin subunit. The Chromatium sp. flavocytochrome is a 72 000 d protein consisting of a 20 000 d dihaem subunit with a redox potential of +20 mV and a 47 000 monoflavin subunit with a redox potential of about +20 mV. The flavin in the

phototrophic bacterial flavocytochrome is FAD, covalently linked to the polypeptide by an 8-α -cysteinyl linkage. The covalently-bound flavin moiety is the immediate acceptor of reducing equivalents from which electrons are rapidly accepted by haem (Cusanovich & Meyer, 1982). Both flavocytochromes have oxidised absorption bands at 410, 450 and 480 nm and reduced absorption bands at 417, 523 and 552 nm (Fig. 5.21) (Pettigrew & Moore, 1987, Wood, 1984; Meyer & Kamen, 1982; Gray & Knaff, 1982).

The plasmid-encoded flavocytochromes of P. putida function as p-cresol methyl hydroxylases and catalyse the 2-electron oxidation of p-cresol, followed by the addition of water to give p-hydroxy benzylalcohol which is then oxidised to p-hydroxy benzaldehyde. The enzymes consist of two 56 000 d subunits, one containing a single haem c with a redox potential of +250 mV and the other containing an o-tyrosyl-linked FAD (Hopper & Taylor, 1977; Kent & Hopper, 1978; Hopper & Kemp, 1980; McIntyre et al., 1981; Hopper et al., 1983).

The flavocytochrome c of S. putrefaciens is different from these cytochromes in a number of respects: it consists of a single polypeptide, the flavin is noncovalently bound, the haems are of very low redox potential, and the cytochrome fails to form the 670 nm "charge transfer complex" with exogenous ligands observed with the phototrophic bacterial

flavocytochromes. These observations would suggest that the flavocytochome c from S. putrefaciens might have a different type of function in vivo from the phototrophic bacterial proteins or the P. putida enzyme.

6.3 Function of the Flavocytochrome c and Peak 8, and Implications for Energy Conservation During Formate Oxidation by TMAO

The presence of flavin as a hydro-dehydrogenation prosthetic group may be physiologically important with respect to loss of protons during transfer of electrons to the haem groups. In a periplasmic flavoprotein the protons would be lost to the outside of the coupling membrane, which is consistent with the chemiosmotic principle. It is noteworthy that no low-potential cytochrome b has been observed in S. putrefaciens. Such cytochromes are typically found associated with formate dehydrogenase in facultative anaerobes where they accept reducing equivalents from formate and conduct electrons to an oxidised redox carrier, leaving protons to be expelled from the enzyme complex to the outer aspect of the inner membrane. The apparent absence of low-potential b-type cytochrome associated with FDH in S. putrefaciens might suggest the presence of an alternative means of separating high-energy reducing equivalents into protons and electrons in such a manner as to expel protons and retain electrons for

further energy conservation steps. Flavocytochrome c appears to be a reasonably good candidate for such a role. It was shown to be formate-reducible, and the redox potentials of the formate/CO_2 couple and the flavocytochrome haems are so close that an intermediate component is unlikely (cytochrome b^{FDH} varies in redox potential: -105 mV for *E. coli* and -200 mV for *W. succinogenes*). The flavin moiety must accept reducing equivalents in the form of hydrogen and must lose protons in order to conduct across itself to the haem groups. The flavocytochrome might thus effectively replace the cytochrome b usually found associated with other formate dehydrogenase complexes. Further evidence for such a role would involve purification and spectral analysis of the formate dehydrogenase, and demonstration of electron transfer from the purified dehydrogenase to the flavocytochrome at rates sufficient to support observed rates of electron transport in the intact system.

The electron acceptor from *S. putrefaciens* flavocytochrome c is unknown. The flavocytochrome was not oxidised by TMAO or by purified TMAO reductase. The simplest explanation is that at least one component mediates electron transport between the flavocytochrome c and TMAO reductase. From thermodynamic considerations and by analogy with other anaerobic electron transport systems (Chapter 1), the most likely candidate might be a flavocytochrome c-menaquinone

oxidoreductase. There are several reasons for suggesting this component. Firstly, the redox potentials of the TMAO/TMA couple (+130 mV) and of ubiquinone/ubiquinol (+113 mV) are very close, particularly when the redox groups of TMAO reductase, which have not yet been fully characterised, are taken into consideration. Thus if a quinone is involved in the formate-TMAO respiratory pathway in S. putrefaciens it is more likely to be menaquinone (-74 mV). Secondly, electron transport from several donors (including NADH and formate) to TMAO was reported to be sensitive to inhibition by the quinone analogue HQNO (Easter, 1982) which is "circumstantial evidence" for the involvement of a quinone and possible b-type cytochromes (see Section 1.2). Thirdly, the involvement of quinone would introduce a site of energy conservation, through the involvement of a proton motive quinone cycle, which may be important in a scheme involving a periplasmic terminal enzyme which catalyses a prototrophic reaction.

Further work on the flavocytochrome might involve identification of the flavin by fluorescence spectroscopy, and measurement of the kinetics of its reactivity with membrane-bound and purified FDH from S. putrefaciens, in conjunction with inhibitor studies and measurements of in vivo formate oxidation. Reconstitution of FDH into proteoliposomes in the absence and presence of flavocytochrome, and

measurements of transmembrane proton gradients resulting from formate pulses, would establish if the flavocytochrome was important for energy conservation as suggested.

The function of Peak 8 is difficult to assess from its physicochemical properties. As a major low-potential cytochrome in S.putrefaciens it was shown to be formate-reducible, but was not reduced directly by formate implying the involvement of other respiratory components between formate and Peak 8. Alternatively it may have been reduced by a low-potential cytochrome active in the formate-TMAO respiratory system by a non-physiological process, made feasible by cell disruption. Given its low redox potential (-200, -300 mV) it is probably reduced directly by a primary dehydrogenase. If this is the case, then it would appear that the primary dehydrogenases of S. putrefaciens as well as the cytochromes are rather different to those found in enteric bacteria since it is implied that these cytochromes (Peak 8 and flavocytochrome) are both replacing the low-potential electron donor components (flavoprotein or cytochrome b) typically found in association with primary dehydrogenases.

The formate dehydrogenase of S. putrefaciens had a high activity when assayed in whole cells using the membrane-impermeant electron acceptor ferricyanide, suggesting that the active site of the enzyme faces the

periplasmic space. It is possible that the organism has developed a system of substrate dehydrogenases adapted to the spoilage environment where substrates normally generated by cytosolic catabolism may be freely available outside the cell. The dehydrogenases would have active sites exposed to the periplasmic space instead of the cytoplasmic aspect of the coupling membrane, which in turn may impose restrictions upon the nature of the prosthetic groups of the dehydrogenases which are reactive with the next component of the respiratory chain. The low-potential periplasmic cytochromes of S. putrefaciens could perform such a role, each cytochrome collecting reducing equivalents from a specific dehydrogenase and delivering them to a common component of the respiratory chain to TMAO. The multihaem nature of flavocytochrome and Peak 8 would support this hypothesis, acting as a redox buffer or store of electrons during diffusion of the cytochromes between the dehydrogenase and the electron acceptor for the cytochromes (Pettigrew & Moore, 1987). This hypothesis could be tested by assaying spheroplasts or whole cells for dehydrogenases in an environment enriched with cytochrome, and also by reconstitution experiments as described above for FDH and flavocytochrome.

Peak 8 shows remarkable similarity to the periplasmic 26 000 d octahaem cytochrome c_3 of Desulfovibrio in terms of optical spectra, redox

potential and number of haem groups. This organism, which grows anaerobically at very low ambient redox potential, utilises the cytochrome as an electron acceptor from periplasmic dehydrogenase. The membrane-permeant hydrogen is derived, in Desulfovibrio, from the cytoplasmic oxidation of pyruvate and lactate (LeGall & Forget, 1978). Interestingly, Desulfovibrio also has a periplasmic formate dehydrogenase of unknown respiratory significance.

E. coli can grow anaerobically using L-malate, fumarate, nitrate, TMAO or protons as electron acceptors (Macey et al., 1976; Bernhard & Gottschalt, 1978; Yamamoto & Ishimoto, 1978). The protons are derived from formate by the cytoplasmic formate hydrogenlyase system, which consists of a formate dehydrogenase which is different from the enzyme involved in nitrate respiration, and a hydrogenase (Ingledew & Poole, 1984), and are used to oxidise excess reductant generated by fermentation. It was mentioned in Section 1.1 that FDH mutants of Salmonella grow well on TMAO whereas formate hydrogenlyase mutants do not (Kwan & Barrett, 1983). Either fhl mutants are pleiotropically affected in anaerobic respiration, or Salmonella degrades formate firstly to CO_2 and H_2, and then uses the H_2 as respiratory substrate via a hydrogenase acting in the reverse direction to the hydrogenase of the E. coli formate hydrogenlyse.

Perhaps S. putrefaciens has two systems for formate degradation; one for exogenous formate using the FDH-flavocytochrome system, and one for endogenous formate using a formate dehydrogenase-hydrogenase-Peak 8 system where formate derived from pyruvate is degraded to $CO_2 + H_2$ in the cytoplasm, and H_2 diffuses across the membrane as in Desulfovibrio to be degraded by hydrogenase and a low-potential cytochrome (Peak 8). Production of gas, however, has not been observed in S. putrefaciens.

A number of areas have been indicated for further work, including examination of the sites and specificity of formate dehydrogenase activity with selected electron acceptors. The determination of quinones in aerobically and anaerobically-grown cells has not yet been carried out, and finally, the nature of labile sulphide in Peak 8 could be determined by measurement of nonhaem iron and labile sulphide.

6.4 The aerobic and anaerobic respiratory chains of S. putrefaciens

6.4.1 The aerobic respiratory system

Four main cytochromes were characterised in aerobically-grown cells of S. putrefaciens: cytochrome $\underline{b}_{560}$, cytochrome $\underline{c}_{552}$ and $\underline{c}_{552,548}$, and cytochrome d. Cytochrome d has been shown to be normally associated with a cytochrome b in those organisms from which it

has been purified; by analogy, the proposed aerobic respiratory system of S. putrefaciens will incorporate a bd complex.

The redox potential of the cytochrome c_{552} (+ 217 mV) is compatible with a proposed function of electron donor to the bd complex. Typically, the small Class I cytochromes c including Pseudomonas c_{551}, with which it is very similar, act as electron donors to terminal respiratory complexes: they are probably reduced by bc_1-type complexes. Results from Section 3.3 indicated that an inhibition site for the FeS-protein inhibitor PCMBS existed between high-potential b- and c-type cytochromes, consistent with a general pattern b_{560} FeS $c_{552,548}$. It would be convenient to suggest that these components might be associated into a complex similar to the bc_1 complex, despite the response to Antimycin A which typically causes an "extra-reduction" of cytochrome b in the complex (Gabellini et al., 1982). Inhibition of reduction of these cytochromes by HQNO (Section 3.1.3) indicates that a quinone is functional between the primary dehydrogenases (for formate and NADH) and the cytochromes. A hypothetical scheme for the aerobic respiratory system of S. putrefaciens is given in Fig. 6.1 which is consistent with these interpretations, and includes the 20 000 d and 11 000 d cytochromes as putative cytochromes c_4 and c_5 donating electrons to cytochrome o as in Azotobacter vinelandii (Section 1.3.3).

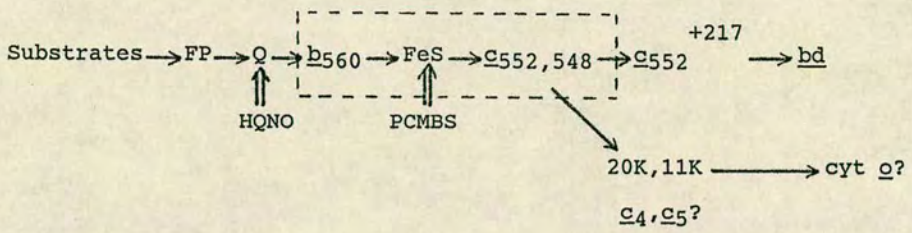


Fig. 6.1 Hypothetical scheme for aerobic electron transport in S. putrefaciens: (i)

This is only one interpretation of the data (cf the E. coli aerobic respiratory system, Section 1.2.2). It is quite possible that the 20K species and a cytochrome c_{548} correlate with $c_{552,548}$ in which case it would seem unlikely that a third high-potential cytochrome would be involved in electron transport from substrate to a single cytochrome oxidase complex. This alternative is given in Fig. 6.2.

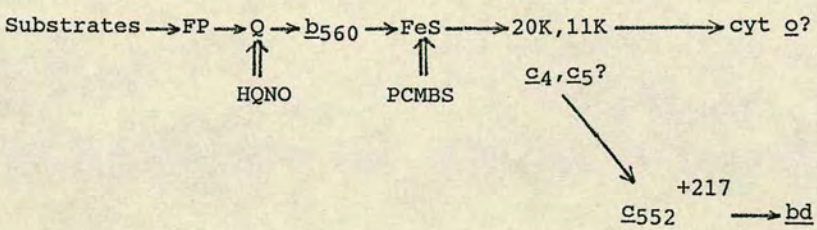


Fig. 6.2 Hypothetical scheme for aerobic electron transport in S. putrefaciens: (ii)

Demonstration of which, if either of these schemes is operating would be achieved by purification of the oxidase complexes and spectral examination of them, in conjunction with kinetic competence studies using their suggested electron donors. Such a study would potentially resolve the structure and function of as many as six cytochromes: cytochrome c (donor) and cytochromes bo or co (oxidase) (if they are present); and cytochrome c (donor) and cytochromes bd (oxidase). It would also possibly resolve the identity of the $c_{552,548}$ species relative to the 20 000 d, 11 000 d and 8 500 d cytochromes, since such a study would necessarily involve separation of membrane-bound components.

The purpose of identifying the aerobic respiratory carriers in S. putrefaciens was to establish if they were also present in anaerobically-grown cells, and if so, to determine if they were involved in TMAO respiration. The aerobic respiratory cytochromes described here were found in microaerobic cells (Section 3.2) and it was demonstrated that they were not involved in TMAO respiration (Section 3.1.4). Hackett & Bragg (1983) found that 50% of the cytochrome content in TMAO-grown cells of E. coli were involved in aerobic respiration, and that these cytochromes were not involved with TMAO respiration (with the possible exception of cytochrome b_{FDH}; see Fig. 1.5). Thus it

might appear that the relatively low redox potential of the TMAO/TMA couple (+ 130 mV) is incompatible with many of the electron transport components common to aerobic respiratory systems, as in fumarate respiration, which separates from the aerobic respiratory systems of many facultative anaerobes at the quinone level (Section 1.2.3).

6.4.2 The Microaerobic Respiratory System

Functions for the flavocytochrome c and Peak 8 from S. putrefaciens were discussed in Section 6.2, as electron acceptors from formate dehydrogenase and hydrogenase, respectively. Both cytochromes were reduced by formate in the presence of FDH-active membrane particles.Both cytochromes were oxidised by TMAO in crude preparations (Section 3.1.4). The flavocytochrome was not oxidised by TMAO or by TMAO reductase, implying the involvement of other components mediating between them (Fig. 6.3).

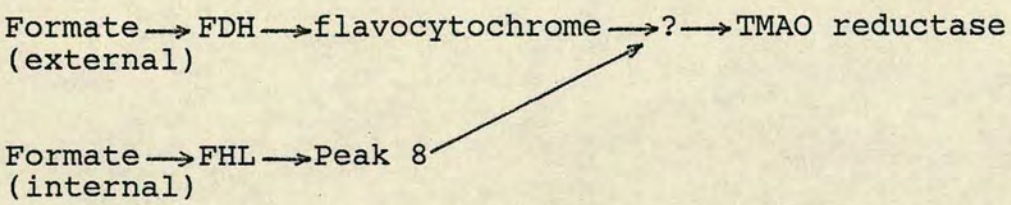


Fig. 6.3 Hypothetical scheme for respiration to TMAO in S. putrefaciens

Also uncertain are the functions of Peak 7, which

had similar redox potentials to Peak 8 and flavocytochrome c, and Peaks 5 and 6, the presence of which was inconsistent in microaerobically-grown cells. It would seem unlikely that each of these cytochromes is specific for a particular dehydrogenase (formate, NADH, lactate). Functions which might be considered for Peaks 5, 6 and 7 are hydroxylamine oxidase and nitrite reductase, both of which are c-type cytochromes in some bacteria (Pettigrew & Moore, 1987).

A large number of cytochromes have been partially characterised in this study and it is now possible to identify each according to its electrophoretic or ionic properties, and to purify them according to the standard procedures described. It is acknowledged however that the results represent a starting point in the determination of the nature and sequence of redox components in the respiratory chains of S. putrefaciens. Where appropriate, the next steps in this direction have been outlined. It appears likely that the purification-reconstitution approach as applied to the aerobic respiratory system of E. coli (Kita et al., 1978; 1984a; 1984b) would be more productive than the inhibitor approach applied successfully to elucidating the formate-fumarate respiratory system of Wolinella succinogenes(Kroger, 1977; 1978; 1980). Of particular significance in this respect are the "competence postulates" described in Section 1.3, which outline the

criteria necessary to show the involvement of a particular protein in a particular electron transport process. Thus a major part of this study was devoted to identifying components common to specific respiratory processes, and to establishing purification/characterisation procedures generally applicable to the cytochromes of S. putrefaciens.

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